Putative cancer stem cells in malignant pleural mesothelioma show resistance to cisplatin and pemetrexed

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Abstract. Malignant pleural mesothelioma (MPM) is a lethal cancer of the mesothelium with high chemotherapeutic resistance via unknown mechanisms. A prevailing hypothesis states that cancer stem cells (CSCs) persist in tumors causing relapse after chemotherapy, thus, rendering these cells as critical targets responsible for tumor resistance and recurrence. We selected candidate CSC markers based on expansion under hypoxic conditions, a hallmark for the selection of chemoresistant cells; and investigated the expression of CSC markers: CD133, Bmi-1, uPAR and ABCG2 in three MPM cell lines and normal mesothelial cells by quantitative RT-PCR. Furthermore, we evaluated the chemotherapeutic resistance associated with each CSC marker by determining the change in CSC marker-mRNA levels as an index of drug-resistance following treatment with either cisplatin or peme-trexed. We demonstrate the expression of CSC markers: CD133, Bmi-1, uPAR and ABCG2 in both normal and MPM cell lines. Bmi-1+, uPAR+ and ABCG2+ cells show a distinct role in conferring chemoresistance to cisplatin and pemetrexed in the malignant setting. By contrast, these markers have no apparent participation in chemoresistance to drug treatments in normal mesothelial cells. Intriguingly, CD133 revealed chemoresistant properties in both normal mesothelial and malignant pleural mesothelioma cells. This study provides evidence of putative CSCs conferring drug-resistance to cisplatin and pemetrexed in MPM cell lines. Specific targeting of these drug-resistant cells, while considering the functional heterogeneity of the MPM subtypes, may contribute to more focused and effective chemotherapeutic regimens for malignant pleural mesothelioma.

Introduction

Malignant pleural mesothelioma (MPM) is a rare but highly aggressive disease with increasing incidence throughout the world (1). This therapy-resistant neoplasm is classified into three histological subtypes: epithelial, sarcomatoid and biphasic (1). At present, there is no curative treatment for MPM, in part, attributed to the drug-resistant properties of tumor cells to chemotherapeutic agents (2). The standard therapeutic approach consists of a combination of chemo-therapy, surgery and radiation; and is often associated with poor prognosis. Pemetrexed and cisplatin combination chemotherapy is the first-line treatment in inoperable disease with a median survival of 9-12.4 months (2). Cisplatin (cis-diamineplatinum dichloride) is a platinum-based chemo-therapeutic drug belonging to the DNA-damaging agents, forming covalent links with DNA (2-4); whereas pemetrexed is a multi-target antifolate agent, active on three enzymes involved in synthesis of thymidine and purine nucleotides (5). Despite notable improvement using multi-modality therapy, disease recurrence and progression remain a problem, thus, it is imperative to determine the cells responsible for the resistance to chemotherapeutic regimens.

Cancer stem cells (CSCs) represent a rare population of cells with stem cell-like characteristics with the capacity to initiate and maintain neoplastic tissues (6,7). Several recent reports have suggested the existence of injury-resistant, CSCs in clinical lung cancer biopsies as well as in established lung cancer cell lines (8-11). Selection of these cells has been primarily based on expression of putative CSC markers which include: CD133, ABCG2, Bmi-1, OCT4 and uPAR. CSCs are more resistant to chemotherapy than the differen-tiated cells of the tumor (12), through their quiescence, ability to repair DNA and adaptation to hypoxic environments (13). Chemoresistance, is in part, mediated by multi-drug resistance ABC transporter proteins (including breast cancer resistant protein, BCRP/ABCG2), which actively, in an ATP-dependent manner extrude a large variety of drugs and drug conjugates from cells (14,15). ABCG2 has been detected in solid tumors including non-small cell lung cancer (NSCLC) in which an involvement in drug resistance to topotecan, and a low response rate to platinum-based therapy have been documented (14).

B lymphoma Mo-MLV insertion region 1, Bmi-1, an oncogenic member of the polycomb repressive complexes (PRCs), has likewise been significantly associated with progression of NSCLC (16,17) and recognized in small cell lung carcinoma (SCLC). Similarly, Urokinase plasminogen activator receptor, uPAR, a membrane glycosylphosphatidyl-inositol anchored 55-60-kDa glycoprotein, correlates with

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poor lung cancer survival and its suppression markedly reduces tumor metastasis (18-20). Rare uPAR+ cells have been identified as putative CSCs in SCLC and overexpression of uPAR has been implicated in an orthotopic murine model of malignant pleural mesothelioma (19).

Recently, the transmembrane glycoprotein, CD133, has been used extensively as a marker of CSCs in cancer tissues. It has been implicated in drug-resistance to chemotherapeutic agents in lung tumor xenografts (8), and in NSCLC tissues and cell lines (9). The latter study highlights the transcription factor, OCT4, as having a pivotal role in maintaining the chemoradioresistant properties of lung cancer CD133+ cells. Embryonic stem cell marker, OCT4 (having the isoforms A and B), has been a focus in cancer stem cell research due to its recent prevalence in malignant settings (9,21,22).

To date, no study has explored the existence of cancer stem cells and/or injury-resistant cells in MPM potentially responsible for the chemotherapeutic resistance and disease recurrence. In this study, we investigated the expression of candidate CSC markers: Bmi-1, uPAR, ABCG2, CD133, OCT4A and OCT4B in three malignant pleural mesothelioma cell lines; and determined the effect of single agent cisplatin and pemetrexed treatment on respective CSC mRNA levels.

Materials and methods

Cell culture. The H28, H2052 and MSTO211H mesothelioma cell lines (LGC Promochem; France) were cultured in RPMI (Invitrogen; Switzerland) media containing 10% fetal bovine serum, FBS (PAA; Austria) and 2% antibiotic/antimycotic (Invitrogen) solution. The non-malignant human pleural mesothelial MeT5A cell line (LGC Promochem) was maintained in Med199 (Sigma; Germany) with 3.3 nM epidermal growth factor (Invitrogen), 400 nM hydrocortisone (Sigma), 870 nM zinc-free insulin (Sigma), 387 ng/l selenious acid (Sigma), 50 ml Trace elements B (1000x) (MediaTech; USA) and 10% FBS. All cell lines were incubated at 37˚C, 95% O_2 and 5% CO_2. Media were replenished every 2 days. For hypoxic culture conditions, cells were incubated for 48 h at 37˚C, 1% O_2, 95% humidity and 5% CO_2 using the Ruskinn hypoxic chamber technology (Ruskinn Tech. Ltd.; Leeds, UK).

XTT assay. Dilution series of 2-fold increments [(0-200 μM; Cisplatin, CDDP, Bristol Myers Squibb, Switzerland) (0-500 μM; Pemetrexed, Eli Lilly, Switzerland)] were prepared for analysis using the XTT cell proliferation assay (Roche Chemicals, Switzerland). To determine the drug-dependent viability, cells (10^4 cells/well in 96-well plates) were incubated in media with or without the addition of drugs. Following a 48 h incubation period, XTT cell proliferation reagent was added according to manufacturer's protocol and formazan production was measured spectrophotometrically (450 nm). Three independent experiments in triplicate were performed.

Drug treatment. For cisplatin treatments, cells were cultured to 80% confluency, followed by treatment with cisplatin at concentrations of 16 μM (H28), 8 μM (H2052), 1 μM (MSTO211H) and 0.3 μM (MeT5A). The specified concentrations correspond to the previously determined IC_{50} values

for each respective cell line. Following the 48 h treatment at 37˚C, media was replenished with growth media in the absence of cisplatin, and cells were allowed to recover for an additional 48 h. A similar protocol was applied for pemetrexed treatment [100 μM (H28 and H2052), 5 μM (MSTO211H) and 0.5 μM (MeT5A)]. Three independent experiments in duplicate were performed for each drug.

Quantitative RT-PCR. Cell samples were collected in TRIzol (Invitrogen) followed by total RNA extraction using RNeasy kit (Qiagen, Switzerland) according to manufacturer's instructions. Complementary DNA, cDNA, was synthesized by reverse transcription (RT) of 5-8 μg of RNA using M-MLV-RT (Promega, Switzerland) and random primers (Roche Diagnostics, Switzerland) and amplified with Taq Polymerase (Roche Diagnostics). Messenger RNA, mRNA, expression levels for house-keeping gene (*β2M*, Hs00984230_m1) and target genes (*OCT4A*, Hs03005111_g1; *OCT4B*, Hs00742896_S1; *CD133*, Hs01009254_m1; *Bmi-1*, Hs00180411_m1; *uPAR*, Hs00182181_m1; and *ABCG2*, Hs01055362_m1) were determined with TaqMan® 'Assay on Demand' probes (Applied Biosystems, Switzerland). cDNAs (50 ng) were subjected to quantitative RT-PCR in a 25 μl reaction volume and analyzed in triplicate. The quantitative RT-PCR data represent the relative quantity of the target gene mRNA compared to the target gene mRNA/*β2M* mRNA ratio determined from the human embryonic carcinoma cell line NTERA-2 (clone D1; European Collection of Cell Cultures, UK), used as a positive control for CSC marker expression. Gene expression was shown as relative amount of mRNA levels using the ΔΔCt method. All mRNA expression levels were based on gene amplifications at <35 cycles, which we considered as a reliable range of amplification.

Statistical analysis. To assess the statistical significance of differences observed in the mRNA expression levels between control and cisplatin-treated or pemetrexed-treated samples, a paired student's t-test was conducted. P-values <0.05 were defined as statistically significant.

Results

Genetic profile of MPM and normal mesothelial cell lines. The presence of potential CSCs in the MPM and control MeT5A cell lines were determined using quantitative RT-PCR for the following markers: Bmi-1, uPAR, ABCG2, CD133, OCT4A and OCT4B. Fig. 1A-D depicts the mRNA expression levels of the markers in each cell line relative to expression levels found in NTERA-2. Equivalent levels of Bmi-1 expression were found in the MPM cell lines, all of which were significantly higher than that observed in MeT5A (Fig. 1E). Similarly, uPAR expression was upregulated in the MPM cells (Fig. 1B) with H2052 cells expressing the greatest levels (Fig. 1B). ABCG2 expression varied amongst the different cell lines with the H2052 cells expressing 3-6 fold higher levels of mRNA in comparison to H28 (Fig. 1A) and MSTO211H (Fig. 1C).

We detected CD133 gene expression in all three MPM cell lines, with a minute level of expression in the control MeT5A cells. Expression of CD133 was significantly higher
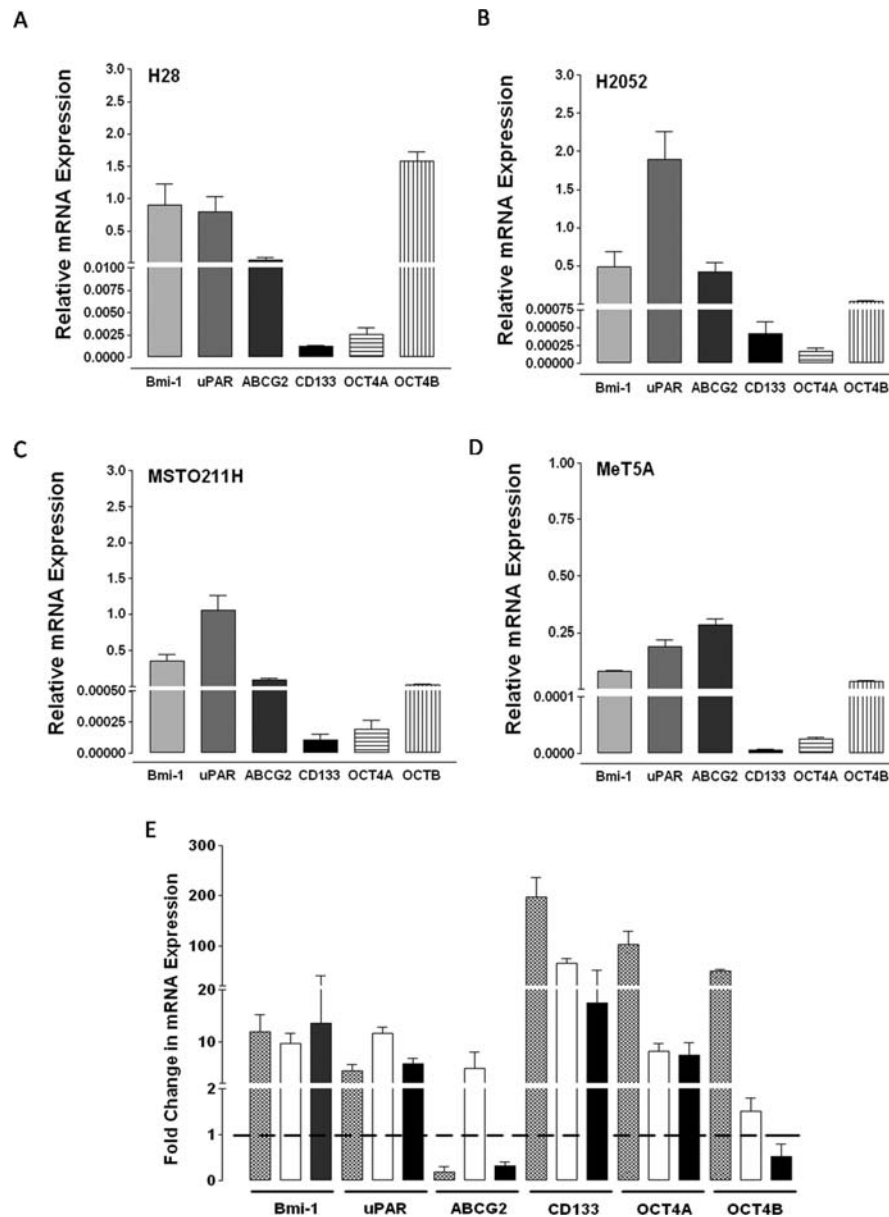
Figure 1. Basal mRNA expression of Bmi-1, uPAR, ABCG2, CD133, OCT4A and OCT4B in MPM cell lines. Quantitative RT-PCR analyses showing the basal mRNA transcript levels in (A) H28, (B) H2052, (C) MSTO211H and (D) MeT5A (control) cell lines under normoxic culture. (E) Fold change in mRNA expression levels of MPM cell lines relative to normal mesothelial MeT5A mRNA values designated at 1 (dashed line) mRNA levels were measured using the ΔΔCt method and relative to values obtained in the NTERA-2 embryonic carcinoma cell line.

in the H28 cell line compared to H2052 and MSTO211H, and was generally expressed at a lower level than the other markers (Fig. 1A-D). Moreover, CD133 mRNA levels were dramatically enhanced in the MPM cell lines (Fig. 1E). In the case of OCT4, established primer/probes designed to specifically target the A and B isoforms, and free from interfering pseudogenes were used (23). OCT4A mRNA levels were highest in H28 cells, and low but detectable in the H2052, MSTO211H and MeT5A cells. In contrast, significantly higher levels of OCT4B (as compared to OCT4A) were observed in all cell lines including MeT5A with no significant rise in expression levels in the MPM cell lines.

Effect of hypoxia on the expression of CSC markers. Hypoxia (low oxygen tension) is a selective insult for genomic alterations that lead to chemotherapeutic resistance observed in

CSC populations (13). Therefore, we selected our candidate CSCs in MPM cell lines (H28, H2052 and MSTO211H) based on CSC expansion under hypoxic culture. Cells were placed under a hypoxic environment for 48 h after which we assessed for changes in CSC mRNA expression levels compared to normal growth under normoxic settings (Fig. 2). As expected, hypoxia induced elevated levels of ABCG2 (24) and CD133 (25). In addition, we detected a significant increase in uPAR expression levels. This is in agreement with recent studies demonstrating enhanced uPAR expression in tumor-derived cells cultured in hypoxic conditions (26). Interestingly, hypoxia had a considerable effect on Bmi-1 expression levels only in the MSTO211H cell line. In contrast to several reports, there was no increase in either OCT4A or OCT4B under hypoxic conditions in any of the MPM cell lines. It is important to note, however, that it is plausible for
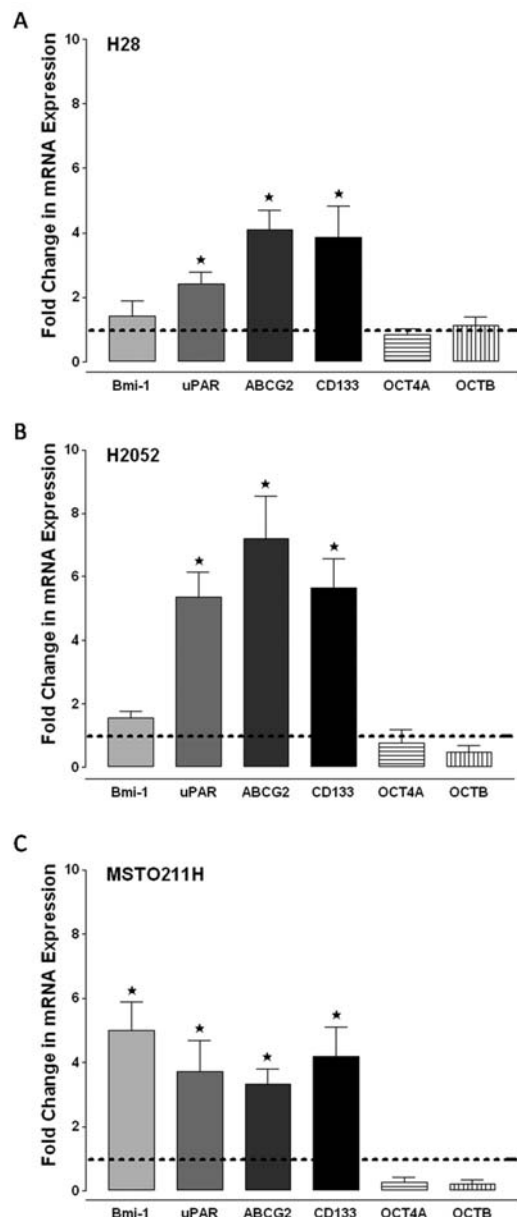
Figure 2. Expression of Bmi-1, uPAR, ABCG2, CD133, OCT4A and OCT4B under hypoxic environment. Quantitative RT-PCR analyses showing the fold changes of mRNA transcripts in H28, H2052 and MSTO211H under hypoxic setting compared to the normoxic basal expression with an assigned value of 1 (dashed line).
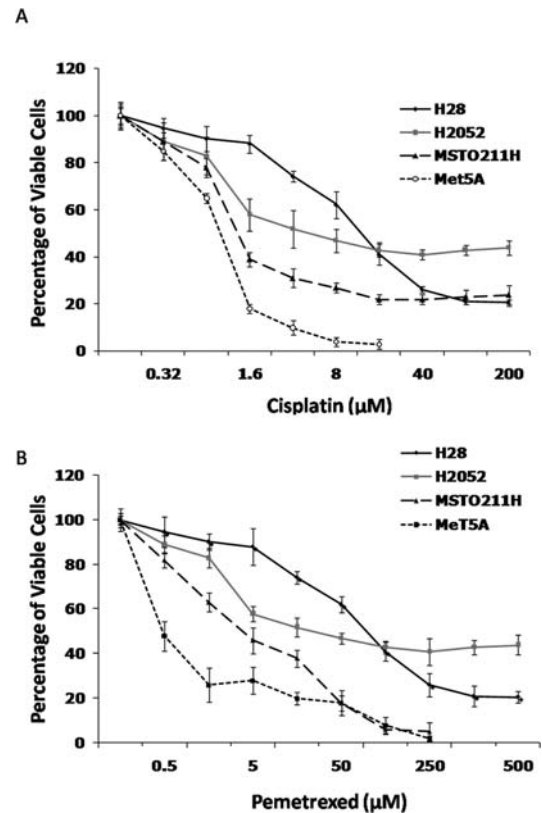

Figure 3. Dose-response curves of MPM cell lines in the presence of either cisplatin or pemetrexed. Effects of different concentrations of (A) cisplatin (0-200 μM) and (B) pemetrexed (0-500 μM) on the cell viability of H28, H2052, MSTO211H and MeT5A as determined by the XTT cell prolife-ration assay. Results represent the means ± SD of three independent experi-ments.

OCT4 to have an oncogenic role in MPM and function as a regulator of chemoradioresistance in cancer stem cell popula-tions (9). Nevertheless, due to the lack of increase of OCT4 expression in MPM cell lines in hypoxic culture, OCT4A and OCT4B were not evaluated in the subsequent drug treatment experiments.

Effect of cisplatin and pemetrexed treatments on cell viabi-lity. To determine the effect of the different concentrations of cisplatin and pemetrexed on cell viability, cells were treated with increasing concentrations of either cisplatin or peme-trexed (Fig. 3). MPM cell lines portrayed relatively variable cell sensitivity levels to cisplatin with an IC_{50} of 16, 8 and 1 μM for H28, H2052 and MSTO211H cells, respectively. Higher resistance to pemetrexed was recognized in all MPM

cell lines cells with an IC_{50} of 100 μM for both H2052 and H28 cells. Note that a much lower IC_{50} (5 μM) was observed for the MSTO211H cell line. MeT5A cells had IC_{50} values of 0.3 μM for cisplatin and 0.5 μM for pemetrexed, respectively.

Effect of cisplatin treatment on CSC expression levels. Cisplatin treatment of H28 cells resulted in a significant increase in uPAR (*p=0.002*) and ABCG2 (*p=0.02*) compared to the non-treated paired controls. In addition, there was a slight increase in Bmi-1 expression although this value did not reach statistical significance. In contrast, there was a significant reduction in CD133 (*p=0.04*) after cisplatin treatment suggesting that CD133+ cells in H28 are sensitive to cisplatin (Fig. 4A). Taken together, these results indicate that in the H28 cell line, ABCG2 and uPAR may be in part, responsible for the resistance to platinum-based chemo-therapeutic drugs.

Despite a slight increase in uPAR expression, treatment of H2052 cells with cisplatin did not show any marked increase in the expression of CSC markers (Fig. 4B). In MSTO211H cells, cisplatin treatment showed an upregulation of Bmi-1 (*p=0.04*), ABCG2 (*p=0.03*) and CD133 (*p=0.001*) (Fig. 4C). Treatment of MeT5A cells resulted in decreased levels of the majority of CSC markers, significantly reducing uPAR (*p=0.0001*) and ABCG2 (*p=0.008*) mRNA levels (Fig. 4D). Interestingly, only CD133 was dramatically increased in the control MeT5A cells suggestive of an innate
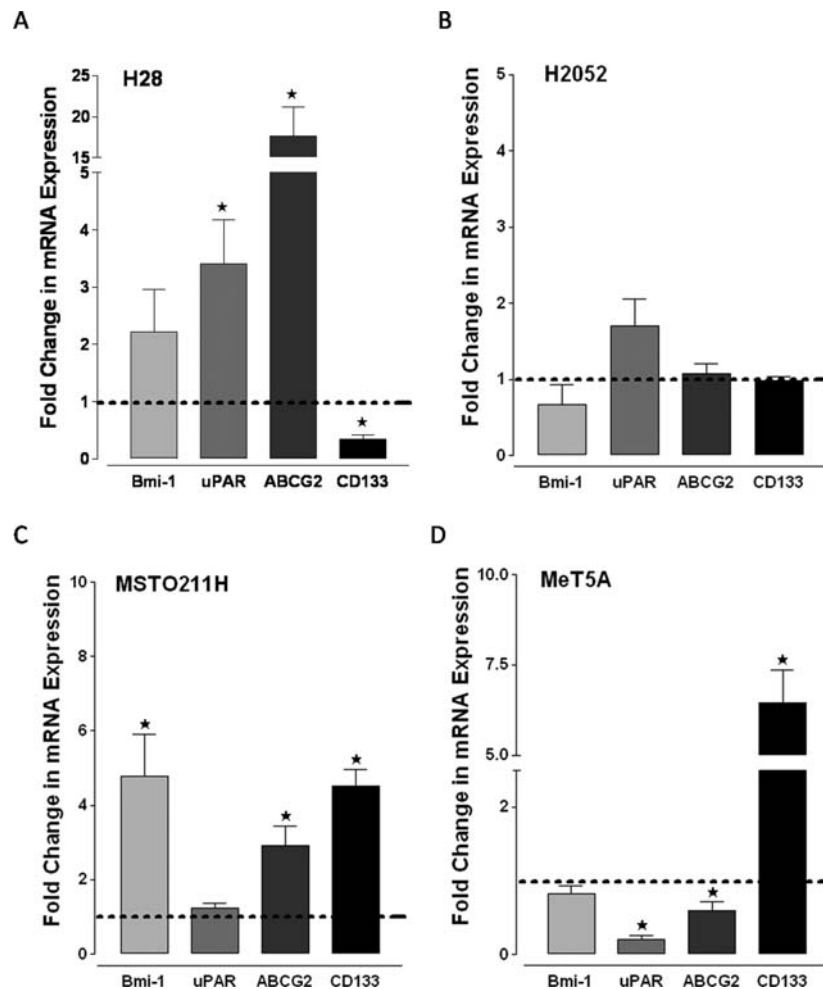
Figure 4. Effect of cisplatin on the expression of candidate CSC markers. Fold changes in the relative mRNA expression of Bmi-1, uPAR, ABCG2 and CD133 following treatments in the absence or presence of cisplatin in (A) H28, (B) H2052, (C) MSTO211H and (D) MeT5A. Non-treated cells were taken as controls with assigned values of 1 (dashed lines). Results represent the means ± SD of a minimum of three independent experiments.

injury-resistive property of CD133+ cells in a non-malignant setting.

Effect of pemetrexed treatment on CSC expression levels. Treatment of H28 cells with pemetrexed resulted in significant elevation of uPAR (*p=0.001*) and ABCG2 (*p=0.0004*) mRNA levels. However, no changes in Bmi-1 and CD133 were observed following pemetrexed treatment (Fig. 5A). Notably, there was a marked increase in CD133 (*p=0.02*) in the H2052 cells indicative of a potential role for CD133+ cells in pemetrexed resistance in this cell line (Fig. 5B). Similar to the effects of cisplatin treatments, results with pemetrexed did not show considerable change in Bmi-1 expression levels in either H28 or H2052 cells. Pemetrexed treatment of MSTO211H cells revealed a significant upregulation of all putative CSC markers (Fig. 5C). Further, pemetrexed treatment of MeT5A cells reduced Bmi-1 (p=0.004) and ABCG2 (p=0.0002) expression and significantly raised CD133 mRNA levels (p=0.00003) (Fig. 5D).

Discussion

In this study, we demonstrate for the first time expression of CSC markers: CD133, Bmi-1, uPAR and ABCG2 in three malignant pleural mesothelioma cell lines H28, H2052 and MSTO211H. We also show that these expression levels are significantly higher than those observed in the non-malignant pleural mesothelial cells, MeT5A. Our data reveal that in normal mesothelial cells, Bmi-1, uPAR and ABCG2 have no apparent participation in chemoresistance to single agent cisplatin and pemetrexed treatments. By contrast, chemotherapeutic treatments convey a distinct role for Bmi-1+, uPAR+ and ABCG2+ cells in conferring chemoresistance to these drugs in the malignant setting. This conforms to the cancer stem cell paradigm indicating the direct participation of CSCs in chemoresistance (27). Intriguingly, CD133 revealed chemoresistant properties in both normal mesothelial cells and malignant pleural mesothelioma cells. In summary, our results suggest the existence of putative drug-resistant CSCs in the three MPM cell lines. Importantly, the degree of drug-resistance elicited by CSCs depends on the specific chemotherapeutic agent used, and the subtype of MPM investigated.

CSCs are hypothesized to be the origin of replicating malignant tumor cells and have been isolated from a variety of solid tumors. The presence of CSCs in tumors may result in enhanced resistance to conventional chemotherapeutic regimens, thus, impairing long-term survival after therapy
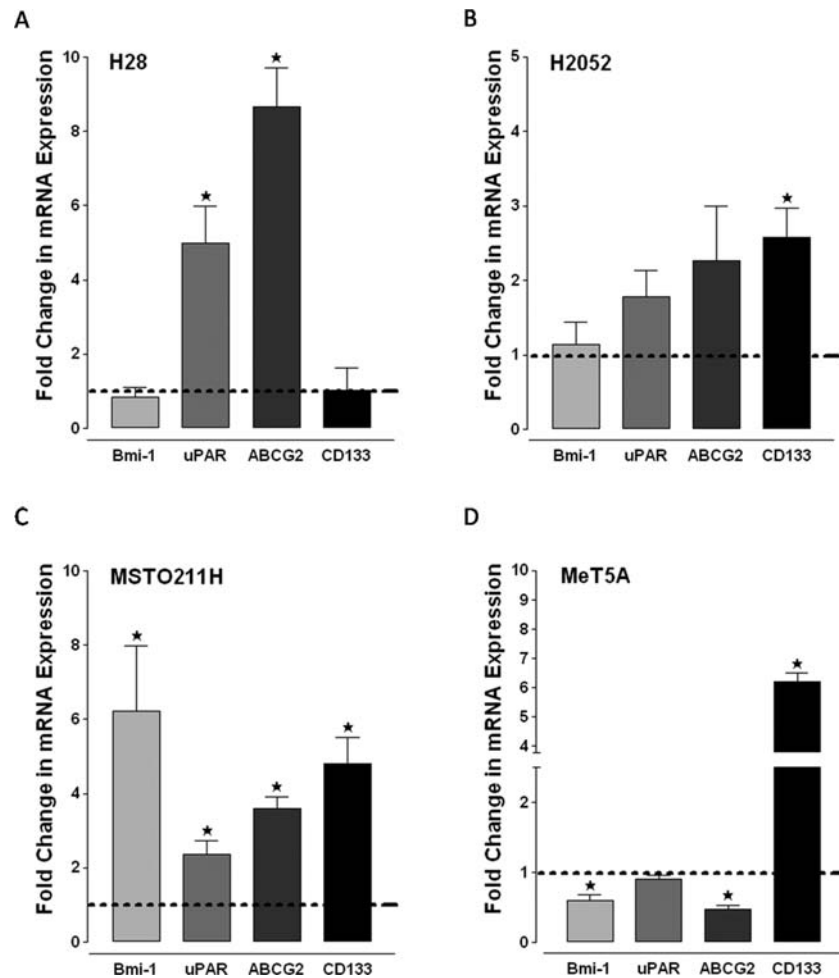
Figure 5. Effect of pemetrexed on the expression of candidate CSC markers. Fold changes in the relative mRNA expression of Bmi-1, uPAR, ABCG2 and CD133 following treatments in the absence or presence of pemetrexed in (A) H28, (B) H2052, (C) MSTO211H and (D) MeT5A. Non-treated cells were taken as controls with assigned values of 1 (dashed lines). Results represent the means ± SD of a minimum of three independent experiments.

(6). In the lung, putative CSC markers include: CD133 (8,10), embryonic stem cell marker OCT4 (9), multi-drug resistant protein ABCG2 (28), Bmi-1 (16, 29) and uPAR (20,30).

Treatment with cisplatin and pemetrexed illustrated a significant increase in ABCG2 expression in H28 and MSTO211H cells inferring a potential role for ABCG2 in the resistance to these chemotherapeutic drugs. Overexpression of ABC transporters reduces the formation of platinum-DNA adducts in tumor cells, resulting in resistance to platinum-based drugs such as cisplatin (31). A study of 156 NSCLC tumors showed that negativity for ABCG2 expression was a positive prognostic factor for advanced NSCLC (32). Likewise, a much lower response rate has been reported following platinum-based chemotherapy in patients with ABCG2 positive tumors (14).

There was no significant ABCG2-mediated drug-resistance following cisplatin and pemetrexed treatments in the H2052 cells. This response to pemetrexed treatment could be attributed to complex pharmacological mechanisms between multi-drug resistant ABC transporters and antifolate properties of the drug. The threshold of ABC transporter expression in pathological conditions is critical for antifolate resistance. High expression of ABCG2, results in increased

folate efflux, thereby leading to reduction of the intracellular folate pool, and a subsequent overall decrease in antifolate resistance (5). The high basal expression of ABCG2 in the H2052 MPM cell could result in reduced intracellular folate pools, and hence, decreased ABCG2-mediated antifolate resistance.

CD133 was first recognized in CD34+ progenitor populations from adult blood and bone marrow and high levels of CD133 expression is now accepted as a characteristic for hematopoietic and neural stem cells (33-35). We detected low levels of CD133 in the MeT5A non-malignant human pleural cells. Further, we found that CD133+ cells are resistant to treatment with chemotherapeutic drugs further supporting CD133 as a potential marker for normal mesothelial progenitor cells with innate injury resistive properties. CD133 has been shown to be highly expressed in CSC populations (6,36-39), and CD133+ CSCs have been deemed to be resistant to chemotherapy treatment (8,10,40). The most recent evidence for CD133 as a CSC marker in the lung was presented by Bertolini *et al* (8) who illustrate a cisplatin-resistant CD133+ subpopulation in primary NSCLC. In agreement with these reports, we observed an upregulation of CD133 in the H2052 and MSTO211H cells following treatment with pemetrexed and in the case of MSTO211H, cisplatin treatment. However,

recent reports have also questioned the validity of CD133 as a marker for cancer stem cells and its functional relevance for tumor-initiation (41). In agreement with these reports, we found that treatments with cisplatin or pemetrexed did not result in elevated CD133 expression levels in H28 cells, and instead, showed a marked CD133 reduction after cisplatin treatment which indicates sensitivity to such treatment.

uPAR is broadly distributed in MPM cells and results in induction of cell response to various stimuli implicated in the pathogenesis of MPM (30). Expression of uPAR has also been found in harvested tissue from MPM patients (30). Here we observe a 4-8 fold increase in basal uPAR mRNA expression levels in the MPM cell lines in comparison to MeT5A cells. This is in agreement with Tucker et al (19) who demonstrate similar levels of increased uPAR protein expression in their tested MPM cell lines. We found that treatment with cisplatin and pemetrexed resulted in a marked increase in uPAR expression in H28 and MSTO211H suggestive of a chemoresistive function of uPAR+ cells in these cell lines. It has been shown that uPAR expression promotes cell survival by activating anti-apoptotic factor Bcl-XL transcription, through the MEK/ERK and PI3K/Akt dependent pathways. Whether the anti-apoptotic mechanism, resulting from increased uPAR expression, confers chemoresistance to the MPM cell lines remains to be investigated.

Bmi-1 has been identified as a member of a death-from-cancer signature profile in a multitude of human cancers and specifically as an oncoprotein in NSCLC (16). Despite its likely oncogenic role in NSCLC, to date, no study has shown Bmi-1 expression in malignant pleural mesothelioma. We found an approximately 5-12 fold increase in basal Bmi-1 mRNA expression levels in the MPM cell lines compared to that in MeT5A. Furthermore, Bmi-1 expression was significantly increased following cisplatin and pemetrexed treatment in MSTO211H cells. Although it has been suggested that elevated expression of Bmi-1 is an early event in the development of lung cancer (16), the exact mechanism of Bmi-1 upregulation still remains to be elucidated. Unlike the MSTO211H cell line, there appears to be no function for Bmi-1 in conferring chemoresistance to cisplatin and pemetrexed in the H28 and H2052 MPM cell lines.

Several recent studies have elucidated functional variances and different responses to chemotherapy between MPM subtypes (42,43). Our results indicate that there are notable differences between epithelial, sarcomatoid and biphasic MPM cell lines with respect to the expression levels of candidate CSC markers, and their response to cisplatin and pemetrexed treatments. It is, therefore, important to take into consideration the cellular and functional heterogeneity of MPM in designing therapeutic regimens for a more effective treatment. This is an important first study, illustrating increased expression levels of putative CSCs with chemoresistive properties in malignant pleural mesothelioma cell lines and warrants further investigation exploring CSC-mediated drug-resistance.

Acknowledgements

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