

Downregulation of membrane complement inhibitors CD55 and CD59 by siRNA sensitises uterine serous carcinoma overexpressing Her2/neu to complement and antibody-dependent cell cytotoxicity *in vitro*: implications for trastuzumab-based immunotherapy

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BACKGROUND: We evaluated the expression of CD46, CD55 and CD59 membrane-bound complement-regulatory proteins (mCRPs) in primary uterine serous carcinoma (USC) and the ability of small interfering RNA (siRNA) against these mCRPs to sensitise USC to complement-dependent cytotoxicity (CDC) and antibody (trastuzumab)-dependent cellular cytotoxicity (ADCC) *in vitro*.

METHODS: Membrane-bound complement-regulatory proteins expression was evaluated using real-time PCR (RT-PCR) and flow cytometry, whereas Her2/neu expression and *c-erbB2* gene amplification were assessed using immunohistochemistry, flow cytometry and fluorescent *in-situ* hybridisation. The biological effect of siRNA-mediated knockdown of mCRPs on HER2/neu-overexpressing USC cell lines was evaluated in CDC and ADCC 4-h chromium-release assays.

RESULTS: High expression of mCRPs was found in USC cell lines when compared with normal endometrial cells ($P < 0.05$). RT-PCR and FACS analyses demonstrated that anti-mCRP siRNAs were effective in reducing CD46, CD55 and CD59 expression on USC ($P < 0.05$). Baseline complement-dependent cytotoxicity (CDC) against USC cell lines was low (mean \pm s.e.m. = $6.8 \pm 0.9\%$) but significantly increased upon CD55 and CD59 knockdown ($11.6 \pm 0.8\%$ and $10.7 \pm 0.9\%$, respectively, $P < 0.05$). Importantly, in the absence of complement, both CD55 and CD59, but not CD46, knockdowns significantly augmented ADCC against USC overexpressing Her2/neu.

CONCLUSION: Uterine serous carcinoma express high levels of the mCRPs CD46, CD55 and CD59. Small interfering RNA inhibition of CD55 and CD59, but not CD46, sensitises USC to both CDC and ADCC *in vitro*, and if specifically targeted to tumour cells, may significantly increase trastuzumab-mediated therapeutic effect *in vivo*.

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Endometrial cancer (EC) is the most common gynaecological malignancy in women, with an incidence of 46 470 new cases and 8120 deaths in 2011 in the United States (Siegel *et al*, 2011). Endometrial cancer is classified based on the clinical picture and histopathological pattern into Type I and Type II disease (Bohkman, 1983; Mutch, 2012). Type I disease includes grade-1 and -2 tumours with endometrioid histology, is oestrogen-dependent and usually preceded by endometrial hyperplasia. This cancer typically occurs in obese patients and is associated with diabetes and hypertension. Most patients are typically diagnosed at an early stage secondary to postmenopausal bleeding and have a good prognosis (Bohkman, 1983; Rose, 1996). In contrast, Type II

EC, which includes uterine serous carcinoma (USC), clear cell cancer and grade-3 endometrioid carcinoma, typically occurs in older thinner patients and is not hormone-dependent (Bohkman, 1983; Mutch, 2012). These tumours are more aggressive and have a worse prognosis than Type I EC (Rose, 1996).

Uterine serous carcinoma represents the most aggressive subtype of EC (Hendrickson *et al*, 1982). Uterine serous carcinoma has been associated with women of African-American ethnicity, tamoxifen use and BRCA gene mutations and is characterised by a high propensity for early lymphovascular invasion, as well as intraperitoneal and extra-abdominal spread at the time of presentation (Hendrickson *et al*, 1982; El-Sahwi *et al*, 2012). The overall 5-year survival is about 30% for all stages and the recurrence rate after surgery is extremely high (50–80%) (Schwartz, 2006; El-Sahwi *et al*, 2012). Thus, there is a dire need for the development of novel, target-specific and more effective therapeutic strategies against this rare subset of EC.

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The *c-erbB2* gene encodes for erbB2 (Her2/neu), a member of the erbB-receptor tyrosine kinase family. This is a family of four transmembrane glycoproteins (erbB1, erbB2, erbB3 and erbB4) that are expressed on epithelial, mesenchymal and neuronal cells. Ligand-binding results in dimerisation of the receptor either with a twin receptor (homodimerisation) or with one of its siblings (heterodimerisation) (Yarden and Sliwkowski, 2001). This leads to phosphorylation of intracellular tyrosine kinase residues that serve as docking sites for effectors and transcription factors that ultimately modulate a variety of biological responses, such as proliferation, survival, migration and differentiation. Our group and others, including the Gynaecologic Oncology Group in cooperative multicenter studies, have reported Her2/neu overexpression (i.e., 2+ and/or 3+ by immunohistochemistry (IHC) in 40–60% of patients harbouring USC (Santin *et al*, 2005a, b, c; Díaz-Montes *et al*, 2006; Morrison *et al*, 2006; Grushko *et al*, 2008). Furthermore, in previous reports, patients with USC overexpressing the Her2/neu receptor have been found to have a worse prognosis than those who do not (Santin *et al*, 2005a, c; Morrison *et al*, 2006). These findings have provided a rationale to the use of Her2/neu-targeted therapies in patients harbouring this aggressive EC subtype.

Trastuzumab (Herceptin; Genentech, San Francisco, CA, USA) is a humanised monoclonal antibody (mAb) directed against the Her2/neu receptor. This mAb has been shown to be highly effective in patients harbouring breast cancer overexpressing Her2/neu (Slamon *et al*, 2001; Baselga *et al*, 2005). Of interest, randomized phase II studies are currently evaluating the potential activity of trastuzumab in combination with chemotherapy in USC patients with advanced/recurrent disease (clinicaltrials.gov/ct2/show/NCT01367002).

Efficacy of cancer immunotherapy with antibodies activating cellular and complement-mediated cytotoxicity is known to be potentially limited by the overexpression of multiple membrane-bound complement-regulatory proteins (mCRPs: CD46, CD55 and CD59) on the surface of tumour cells (Gelderman *et al*, 2004, 2005). However, to our knowledge, no study has yet investigated whether biologically aggressive USC may overexpress mCRPs and whether downregulation of CD46, CD55 and CD59 by small interfering RNA (siRNA) may sensitise USC overexpressing HER2/neu to complement and/or trastuzumab-dependent cell-mediated cytotoxicity. To fill this gap in knowledge, in this study we analysed 15 primary USC cell lines for CD46, CD55, CD59, Her2/neu receptor expression and *c-erbB2* gene amplification and investigated the ability of siRNA against these mCRPs to sensitise USC to complement and antibody (trastuzumab)-induced cellular cytotoxicity *in vitro*.

MATERIALS AND METHODS

Establishment of USC cell lines

A total of 15 primary USC cell lines (USPC-ARK-1–USPC-ARK-15) were established after sterile processing of tumour samples from surgical biopsy specimens, as described previously (El-Sahwi *et al*, 2010), under approval of the Institutional Review Board. Tumours were staged according to the International Federation of Gynecologists and Obstetricians 1988 operative staging system. Source-patient characteristics of these 15 USC cell lines are described in Table 1.

Her2/neu immunostaining of formalin-fixed tumour tissues

Formalin-fixed, paraffin-embedded tissue blocks from the USC patients from whom primary cell lines were established were retrieved from surgical pathology files. Specimens were reviewed by a gynaecologic pathologist. The level of Her2/neu expression was evaluated on the most representative block by the standard

immunohistochemical staining, using the Hercept test (Dako, Glostrup, Denmark), as previously described (El-Sahwi *et al*, 2010).

Fluorescent *in-situ* hybridisation (FISH)

Fluorescent *in-situ* hybridisation analysis was performed on either cell blocks or formalin-fixed paraffin-embedded tissue blocks from all USCs using the PathVysion Her-2 DNA FISH Kit (Abbott Molecular Inc., Abbott Park, IL, USA) according to the manufacturer's instructions, as previously described (El-Sahwi *et al*, 2010).

Quantitative real-time PCR (qRT-PCR)

RNA isolation from all the 15 primary USC cell lines was performed using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Quantitative PCR was carried out with a 7500 RealTime PCR System using the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA, USA) to evaluate the expression of CD46, CD55 and CD59 mCRPs in all samples. The primers and probe for CD46, CD55 and CD59 were obtained from Applied Biosystems (i.e., CD46 Assay ID: Hs00611257_m1, CD55 Assay ID: Hs00892618_m1 and CD59 Assay ID: Hs00174141_m1). The comparative threshold cycle method was used to determine gene expression in each sample, relative to the value observed in non-malignant endometrial epithelial cell samples collected from similar-age women, using glyceraldehyde-3-phosphate dehydrogenase (Assay ID Hs99999905_m1) RNA as an internal control.

Small interfering RNA knockdown experiments

CD46-, CD55- and CD59-specific siRNA oligonucleotides (i.e., CD46: 5'-GGAUACUUCUAUAUACCUCUU-3' (sense) and 3'-UUCCUAUGAAGAUUAUUGGAG-5' (antisense); CD55: 5'-AUGU GAAGAAAGCUUUGUGUU-3' (sense) and 3'-UUUACACUUCUU UCGAAACAC-5' (antisense); CD59: 5'-GGACCUGUGUAACUUU AACUU-3' (sense) and 3'-UUCCUGGACACAUUGAAAUUG-5' (antisense)) and nonspecific siRNA duplexes used as negative controls, were purchased from Ambion, Inc. (Austin, TX, USA). Briefly, representative USC cell lines harbouring amplification of the *c-erbB2* gene were cultured in six-well plates and transfected with anti-CD46, anti-CD55 or anti-CD59 siRNA duplexes at 10 nM in conjunction with 5 µl Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions. Mock transfections and nonspecific siRNA duplexes were used as negative controls. USC cells were treated for 72 h (i.e., the time we found required for maximal downregulation of CD46, CD55 or CD59, based on flow cytometry and qRT-PCR), after which they were used in CDC and antibody (trastuzumab)-dependent cellular cytotoxicity (ADCC) as described below.

Flow cytometry

The clinically marketed anti-Her2/neu trastuzumab (Herceptin; Genentech), a humanised mAb of the G1 type that binds with high affinity to the extracellular domain of the HER2/neu receptor and commercially available antibodies against mCRPs CD46 (cat. no. 555948), CD55 (cat. no. 555691) and CD59 (cat. no. 555761) (BD Pharmingen, San Diego, CA, USA), were used for our study. Primary USC cell lines obtained from the above described patients were stained with 2.5 µg ml⁻¹ of trastuzumab or 5 µg per 200 µl of anti-CD46, anti-CD55 or anti-CD59 for 30 min on ice. A total of 5 µg ml⁻¹ of the chimeric anti-CD20 mAb rituximab (Rituxan; Genentech) was used as a negative control. Fluorescein isothiocyanate-conjugated goat antihuman F(ab)2 immunoglobulin (BioSource International) or goat-anti-mouse FITC (BD Pharmingen) were used for staining as a secondary reagents.

Table 1 Patient characteristics from which the 15 USC cell lines were established

| Patient | Age | Race | Stage | Histology | IHC Her2/neu | FISH Her2/neu |
|-------------|-----|------|-------|-----------|--------------|---------------|
| USPC ARK-1 | 62 | AA | IVA | Pure | 3+ | Positive |
| USPC ARK-2 | 63 | AA | IVB | Pure | 3+ | Positive |
| USPC ARK-3 | 59 | AA | IVB | Mixed | 3+ | Positive |
| USPC ARK-4 | 73 | C | IVB | Pure | 1+ | Negative |
| USPC ARK-5 | 73 | AA | IIIC | Pure | 1+ | Negative |
| USPC ARK-6 | 62 | C | IB | Mixed | 1+ | Negative |
| USPC ARK-7 | 75 | C | IIC | Pure | 2+ | Negative |
| USPC ARK-8 | 88 | C | IIIA | Pure | 1+ | Negative |
| USPC ARK-9 | 73 | AA | IIIC | Mixed | 3+ | Positive |
| USPC ARK-10 | 79 | C | IVB | Pure | 3+ | Positive |
| USPC ARK-11 | 80 | AA | IIIC | Mixed | 1+ | Negative |
| USPC ARK-12 | 64 | C | IVB | Pure | 0 | Negative |
| USPC ARK-13 | 67 | C | IVB | Mixed | 2+ | Negative |
| USPC ARK-14 | 73 | AA | IV | Pure | 0 | Negative |
| USPC ARK-15 | 67 | C | IIIC | Pure | 1+ | Negative |

Abbreviations: AA = African American; C = Caucasian; USPC = uterine serous papillary adenocarcinoma.

Analysis was conducted with a FACScalibur, using Cell Quest software (BD Biosciences).

Tests for ADCC

A standard 4-h chromium (^{51}Cr)-release assay was performed to measure the cytotoxic reactivity of Ficoll-PaqueTM PLUS-separated (GE Healthcare, Uppsala, Sweden) peripheral blood lymphocytes (PBLs) obtained from several healthy donors against USPC cell lines harbouring amplification of the *c-erbB2* gene before and after knockdown in CD46, CD55 and CD59 expression by siRNA. The release of ^{51}Cr from the target cells was measured as evidence of tumour cell lysis after exposure of tumour cells to $5\ \mu\text{g ml}^{-1}$ of trastuzumab. Additional controls included the incubation of target cells alone or with PBL or mAb separately. The chimeric anti-CD20 mAb rituximab was used as a negative control for trastuzumab in the bioassays. antibody (trastuzumab)-dependent cellular cytotoxicity was calculated as percentage killing of target cells obtained after trastuzumab incubation with effector cells compared with ^{51}Cr release from target cells incubated alone.

Complement-mediated target cell lysis of USC cell lines

A standard 4-h ^{51}Cr -release assay identical to the one used for ADCC assays was used, except that human plasma in a dilution of 1:2 was added in place of the effector cells in AIM-V culture medium (Invitrogen). This human plasma was used as a source of complement to test for complement-mediated target cell lysis before and after knockdown in CD46, CD55 and CD59 expression by siRNA in the presence of trastuzumab. In additional experiments, heat-inactivated ($56\ ^\circ\text{C}$ for 60 min) human plasma was diluted 1:2 before addition to the assay. Rituximab was used as isotype control mAb. In all experiments, controls included the incubation of target cells alone or with mAb separately.

Statistical analysis

For qRT-PCR data, the right-skewing was removed by taking copy number ratios relative to the lowest-expressing normal human EC sample ('relative copy number'), \log_2 transforming them to ΔC_t s and comparing the results through unequal variance *t*-test for FISH-positive vs FISH-negative USC. Group means with 95% confidence intervals (CIs) were calculated by computing them on the ΔC_t s and then reverse-transforming the results to obtain means (95% CIs) of relative copy numbers. The analyses of differences in mCRP expression levels after knockdown of CD46, CD55 and CD59 by siRNA measured by RT-PCR and flow cytometry were performed using the Wilcoxon Mann-Whitney test. Kruskal-Wallis

test and χ^2 analysis were used to evaluate differences in CDC and trastuzumab-mediated ADCC levels in primary tumour cell lines before and after knockdown of CD46, CD55 and CD59 by siRNA. Statistical analysis was performed using PASW version 18 (SPSS, Chicago, IL, USA) and GraphPad Prism (GraphPad Software, Inc., LaJolla, CA, USA). A *P*-value of <0.05 was considered statistically significant.

RESULTS

Her2/neu expression by IHC on USC

Immunohistochemistry detecting Her2/neu expression was performed on formalin-fixed paraffin-blocks of USC tissues from which the 15 primary cell lines were established. As reported in Table 1, 5 out of 15 specimens showed strong staining (3+) for Her2/neu protein, whereas the remaining 10 showed weak to moderate staining (0, 1+ and 2+).

Fluorescent *in-situ* hybridisation

Fluorescent *in-situ* hybridisation analysis was performed on either cell blocks or formalin-fixed paraffin-embedded tissue blocks from all USCs used in this study. *c-erbB2* gene amplification was detected in all five primary USC specimens showing 3+ positive expression by IHC (Table 1), suggesting that strong receptor expression by IHC and high Her2/neu mRNA level of these tumours (see below) is likely caused by gene amplification. In contrast, the remaining 10 USC cell lines were found to be negative for *c-erbB2* gene amplification (Table 1).

Flow cytometry

Surface Her2/neu expression was evaluated by FACS analysis on all the 15 primary USC cell lines using trastuzumab. In addition, as negative controls, several B cell lines (EBV-transformed lymphoblastoid B cell lines) established from the same USC patients from which the tumour cell lines had been established were also studied (data not shown). In all, 4 out of 15 USC cell lines (all FISH positive) showed a very high expression of Her2/neu (mean fluorescence intensity (MFI) ranging from 228 to 339), whereas the remaining 11 (1 FISH positive and 10 FISH negative) were found to express significantly lower levels of Her2/neu (MFI ranging from 10 to 72) (Table 2, *P* > 0.01).

Table 2 Complement regulatory protein and Her2/neu expression by flow cytometry in uterine serous tumours

| Cell lines | CD46 | | CD55 | | CD59 | | Her2/neu | |
|-------------|------|-----------------|------|-----|------|-----|----------|-----|
| | % | MFI | % | MFI | % | MFI | % | MFI |
| USPC-ARK-1 | 99 | 54 ^a | 98 | 43 | 100 | 102 | 100 | 339 |
| USPC-ARK-2 | 99 | 64 | 100 | 59 | 99 | 45 | 100 | 230 |
| USPC-ARK-3 | 100 | 64 | 100 | 71 | 100 | 55 | 100 | 228 |
| USPC-ARK-4 | 100 | 51 | 100 | 73 | 100 | 160 | 95 | 10 |
| USPC-ARK-5 | 98 | 33 | 98 | 52 | 95 | 32 | 84 | 18 |
| USPC-ARK-6 | 100 | 70 | 98 | 37 | 98 | 39 | 93 | 13 |
| USPC-ARK-7 | 100 | 24 | 53 | 20 | 100 | 46 | 95 | 47 |
| USPC-ARK-8 | 100 | 115 | 100 | 97 | 100 | 84 | 83 | 24 |
| USPC-ARK-9 | 99 | 123 | 99 | 107 | 99 | 85 | 99 | 285 |
| USPC-ARK-10 | 100 | 54 | 100 | 46 | 100 | 57 | 99 | 25 |
| USPC-ARK-11 | 100 | 163 | 99 | 67 | 99 | 123 | 85 | 23 |
| USPC-ARK-12 | 99 | 49 | 100 | 59 | 58 | 19 | 99 | 32 |
| USPC-ARK-13 | 95 | 48 | 91 | 35 | 64 | 22 | 100 | 11 |
| USPC-ARK-14 | 99 | 60 | 100 | 61 | 85 | 31 | 99 | 26 |
| USPC-ARK-15 | 99 | 45 | 99 | 50 | 57 | 20 | 100 | 72 |

Abbreviations: MFI = mean fluorescence intensity; USPC = uterine serous papillary adenocarcinoma. ^aFlow cytometry results are representative of MFI values of a minimum of three independent experiments for USC cell line.

qRT-PCR

A total of 15 primary USC cell lines were tested by RT-PCR for the expression of CD46, CD55 and CD59 mCRP at mRNA level. Expression for CD46, CD55 and CD59 mCRP was detected in all 15 USC cell lines tested. CD46, CD55 and CD59 mRNA transcripts were 17.6 ± 3.8, 3.0 ± 1.0 and 7.2 ± 1.3 fold higher, respectively, (mean ± s.e.) when compared with the level of expression found in normal control endometrial cells (P < 0.05). No significant differences in mCRP expression for CD46, CD55 and CD59 were detected when USC cell lines with high HER2/neu expression (i.e., FISH positive) were compared with those with low HER2/neu expression (i.e., FISH negative, data not shown).

mCRP expression by flow cytometry

To determine whether the expression of CD46, CD55 and CD59 mCRP mRNA detected using qRT-PCR in USC cell lines also resulted in expression of the protein on the surface of tumour cells, flow cytometry was performed on all primary cell lines. Membrane-bound complement-regulatory proteins surface-expression results from flow-cytometry analysis were found to be in good agreement with mCRP expression results found by qRT-PCR in primary cell lines (Table 2). No significant differences were found between high vs low HER2/neu expressor USC cell lines for any of the mCRP tested (Table 2 and data not shown).

Downregulation of mCRP expression by anti-CD46, anti-CD55 and anti-CD59 siRNA

Uterine serous carcinoma cell lines harbouring amplification of c-erbB2 by FISH were transfected with selected siRNA specific for CD46, CD55 and CD59 and inhibition of individual mCRP knockdown was evaluated by FACS analysis and RT-PCR at different time points. We found the best inhibition rates for anti-CD46, anti-CD55 and anti-CD59 siRNA at 72 h after transfection (data not shown). Upon optimisation, CD46 protein expression was decreased by siRNA by 83% in USPC-ARK-2 (Figure 1) and by 71% in USPC-ARK-3 (data not shown). CD55 protein expression was decreased by siRNA by 51% in USPC-ARK-2 and by 53% in USPC-ARK-3, whereas CD59 protein expression was decreased by

siRNA by 92% in USPC-ARK-2 and by 93% in USPC-ARK-3 (P < 0.05) (Figure 1). qRT-PCR analysis at 72 h after transfection with siRNA specific for CD46, CD55 and CD59 confirmed the results obtained at protein levels by flow cytometry. Specifically, we found a mean downregulation of CD46 expression by siRNA by 86% in USPC-ARK-2 and by 93% in USPC-ARK-3. CD55 expression was decreased by siRNA by 77% in USPC-ARK-2 and by 78% in USPC-ARK-3, whereas CD59 expression was decreased by siRNA by 97% in USPC-ARK-2 and by 98% in USPC-ARK-3 (P < 0.05).

Effects of knockdown of CD46, CD55 and CD59 by siRNA on CDC in USC cell lines overexpressing HER2/neu

In order to evaluate the effect of complement against USC cell lines, human plasma diluted 1:2 (with and without heat inactivation) was added during the standard 4-h ⁵¹Cr-release cytotoxicity assays against USPC-ARK-2 and USPC-ARK-3 tumour cell lines expressing high levels of Her2/neu before and after knockdown of CD46, CD55 and CD59 by siRNA. As representatively shown in Figure 2, for both USC cell lines, in the absence of PBL the addition of untreated plasma in the presence of trastuzumab induced low levels of cytotoxicity against the USC cell lines (mean ± s.e.m. = 6.8% ± 0.9%). Importantly, downregulation of CD55 and CD59, but not CD46, significantly increased CDC against both tumours. Mean killing ± s.e.m. after knockdown of CD55 and CD59 was 11.6 ± 0.8% and 10.7 ± 0.9%, respectively, (Figure 2, P < 0.05). Inactivation of human plasma by heat reduced CDC to negligible levels (i.e., < 1%, data not shown). These results demonstrating the importance of CD55 and CD59 expression in potentially inhibiting complement attack against USC cell lines overexpressing Her2/neu in the presence of trastuzumab. In additional experiments, we evaluated the effect of the simultaneous block of all three mCRP by anti-CD46, anti-CD55 and anti-CD59 siRNA against USPC-ARK-2 and USPC-ARK-3 tumour cell lines in CDC experiments. Mean killing ± s.e.m. after the simultaneous knockdown of CD46, CD55 and CD59 was 13.6 ± 1.8% and 11.9 ± 1.1% in USPC-ARK-2 and USPC-ARK-3, respectively (P < 0.05).

Effects of knockdown of CD46, CD55 and CD59 by siRNA on ADCC in USC cell lines overexpressing HER2/neu

Two representative primary USC cell lines (i.e., USPC-ARK-2 and USPC-ARK-3) harbouring amplification of the c-erbB2 were tested for their sensitivity to natural killer (NK) cytotoxicity when challenged with heterologous PBL, collected from several healthy donors, in a standard 4-hr ⁵¹Cr-release assay before and after knockdown of CD46, CD55 and CD59 by siRNA in the absence of plasma as source of complement. Both cell lines were found to be highly resistant to NK-mediated cytotoxicity when combined with PBLs at effector to target cell ratios (E:T) varying from 10:1 to 20:1 in the absence of trastuzumab (mean killing ± s.e.m. = 3.0% ± 2.1 with all E:T). Similarly, both USC cell lines incubated with rituximab control antibody and PBL were not significantly killed (mean killing ± s.e.m. = 2.9 ± 2.0% with all E:T, data not shown). In contrast, as demonstrated in Figure 3, USPC-ARK-2 and USPC-ARK-3 were highly sensitive to trastuzumab-mediated ADCC (mean killing ± s.e.m. = 48.0 ± 1.1% at 20:1 ratio). We then evaluated the sensitivity of USPC-ARK-2 and USPC-ARK-3 cell lines to NK cytotoxicity and trastuzumab after knockdown of CD46, CD55 and CD59 by siRNA. We found downregulation of CD55 and CD59 to consistently and significantly increase NK-mediated killing in both the cell lines in multiple experiments (mean killing ± s.e.m. = 62.5 ± 2.1% and 64.8 ± 2.1% at 20:1 ratio, respectively, P < 0.05, Figure 3). In contrast, downregulation of CD46 by siRNA did not significantly alter NK-mediated killing in

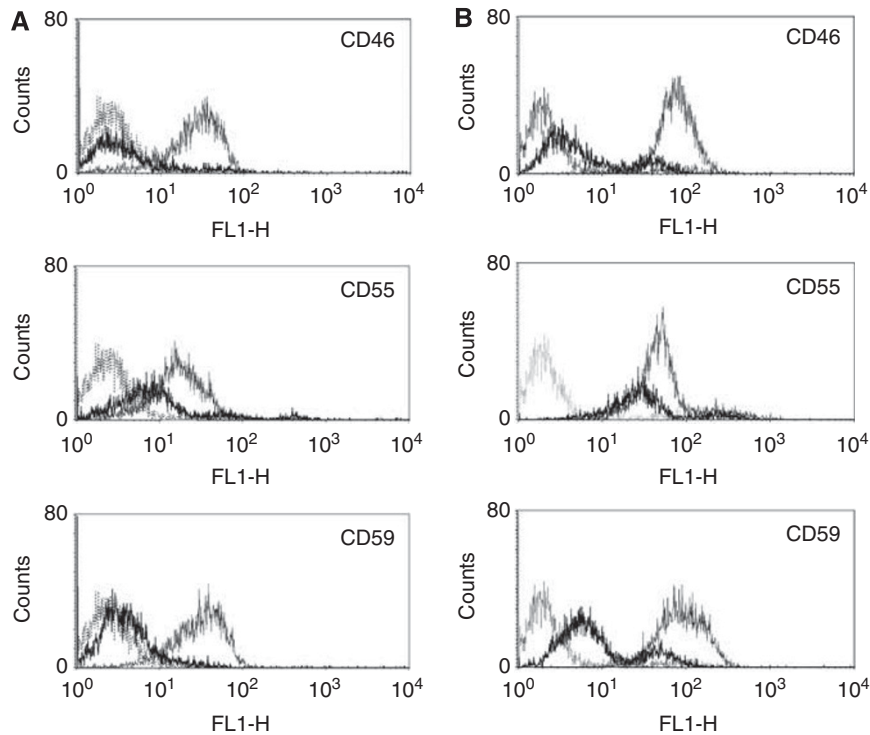


Figure 1 (A) Representative FACS histograms showing knockdown of the mCRPs CD46, CD55 and CD59 in uterine serous papillary carcinoma cell line USPC-ARK-2. (B) Representative FACS histograms showing knockdown of the mCRPs CD46, CD55 and CD59 in uterine serous papillary carcinoma cell line USPC-ARK-3. The faint line depicts CD46, CD55 and CD59 expression in USPC cell lines before transfection with each mCRP-specific siRNA. The bold line describes CD46, CD55 and CD59 expression after transfection with each mCRP-specific siRNA. Dotted line: isotype control. A significant downregulation of mCRP expression by anti-CD46, anti-CD55 and anti-CD59 siRNA was noted in both cell lines tested ($P < 0.05$).

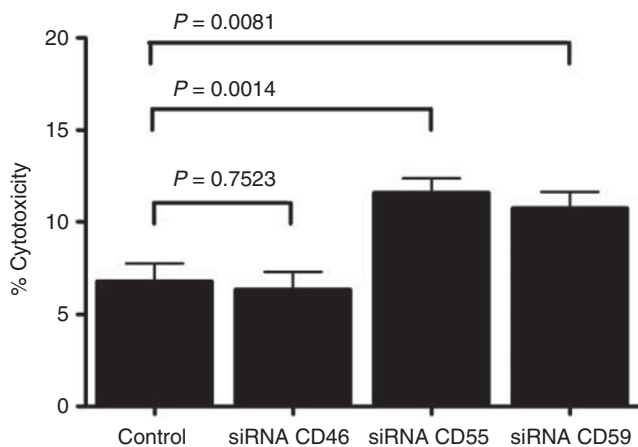


Figure 2 Pooled cytotoxicity data in the setting of siRNA against CD46, CD55 and CD59 in uterine serous papillary carcinoma. Silencing of CD55 and CD59, but not CD46, augments CDC. Mean \pm s.e.m. are shown representing 10 replicates using two cell lines. Controls in the experiments included both mock-transfected and USC cells pretreated with nonspecific siRNA as described in the methods section.

either cell line (mean killing \pm s.e.m. = $45.9 \pm 0.7\%$ at 20:1 ratio, Figure 3).

DISCUSSION

Resistance of tumour cells to lysis mediated by NK cells and homologous complement by upregulation of mCRP, such as

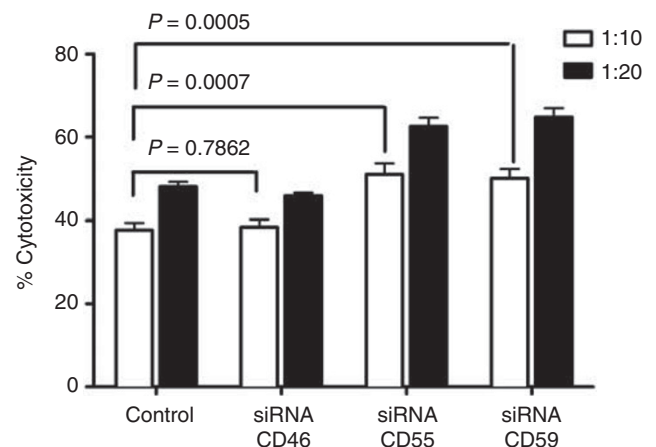


Figure 3 Pooled antibody-dependent cell-mediated cytotoxicity at effector to target ratios of 10:1 and 20:1 in the setting of siRNA against CD46, CD55 and CD59 in uterine serous papillary carcinoma. Silencing of CD55 and CD59, but not CD46, enhances trastuzumab-dependent cell-mediated cytotoxicity at all concentrations examined. Mean \pm s.e.m. are shown representing 18 replicates across two cell lines. P -values for 10:1 ratios are provided; corresponding P -values for control vs siRNA CD46, siRNA CD55 and siRNA CD59 at 20:1 are 0.1225, < 0.0001 , < 0.0001 , respectively.

membrane cofactor protein (CD46), decay-accelerating factor (CD55) and protectin (CD59), are potentially major immune-escape strategies developed by tumours (reviewed in Gelderman *et al*, 2004, 2005). Indeed, in many tumours, mCRP expression is

greater than in normal surrounding tissues and mCRP act as physiological brakes to complement amplification on normal as well as tumour cells either by limiting formation of the C3/C5 convertase enzymes (i.e., CD46 and CD55) or by assembly of the cytolytic membrane-attack complex (MAC, i.e., CD59) (reviewed in Gelderman *et al*, 2004, 2005). Consistent with this view, cells from patients with the haematological disorder paroxysmal nocturnal hemoglobinuria are defective in the machinery for glycosylphosphatidylinositol-anchor synthesis and hence lack surface expression of both CD55 and CD59 and are highly susceptible to the lytic effect of MAC (Iida *et al*, 1994). Importantly, *in vitro* blocking studies of CD55 and CD59 by antibodies or miniantibodies have been reported to increase not only complement-mediated lysis but also antibody-dependent cell-mediated cytotoxicity against multiple human and rat tumours in the absence of complement (Finberg *et al*, 1992; Caragine *et al*, 2002; Macor *et al*, 2007). These findings suggesting additional noncomplement-dependent function for these mCRP are noteworthy because, although multiple mechanisms of action have been attributed to clinically approved antibodies targeting specific targets on human tumours such as trastuzumab (i.e., anti-HER2/neu), rituximab (i.e., anti-CD20) or cetuximab (i.e., anti-EGFR), including inhibition of tumour proliferation, angiogenesis and metastasis, induction of apoptosis and/or promotion of cell cycle arrest, strong experimental evidence currently suggests that engagement of Fc receptors on effector cells (i.e., mainly NK cells) represents the dominant component of the *in vivo* activity of these mAbs against human tumours (Clynes *et al*, 2000). In this regard, NK cells are considered the best-suited lymphocytes for ADCC because they carry Fc γ RIII receptors, which are activator molecules, and not Fc γ RIIb receptors, which inhibit ADCC (Clynes *et al*, 2000). In support of this hypothesis, the efficacy of the anti-EGFR antibody (M225) when used as an F(ab)₂ in blocking the growth of tumours *in vivo* has been previously demonstrated to be only 50% of the activity shown by the intact antibody when it is able to engage the Fc receptors on NK cells (Fan *et al*, 1993). Similarly, the engagement of Fc γ RIII receptors on NK effector cells has been demonstrated to represent the dominant component of the *in vivo* activities of trastuzumab and rituximab in multiple animal models (Clynes *et al*, 2000). On the basis of this evidence, it seems therefore likely that specific inhibition of mCRP, such as CD46, CD55 and CD59, on USC harbouring amplification of the *c-erbB2* gene may have the potential to sensitise further these biologically aggressive tumours to trastuzumab-mediated CDC and ADCC.

In the current study, using multiple techniques, we demonstrated high Her2/neu expression in 5 out of 15 primary USC cell lines established in our laboratory. We also confirmed that USC cell lines overexpressing Her2/neu are sensitive to NK-mediated cytotoxicity in the presence of trastuzumab (El-Sahwi *et al*, 2010). Importantly, because of the lack of information regarding expression of mCRP on USC, we carefully investigated CD46, CD55 and CD59 expression in all the 15 primary USC cell lines and analysed whether siRNA against CD46, CD55 or CD59 may sensitise USC to CDC and ADCC *in vitro*. We report for the first time increased expression of mCRP in all primary USC available to us (i.e., 15 primary USC cell lines) regardless of their high or low HER2/neu expression. These results are consistent with the upregulation of CD46, CD55 or CD59 mCRP previously reported in various human tumours, including colorectal cancer, cervical cancer, prostate cancer and renal cell carcinoma (reviewed in Gelderman *et al*, 2004, 2005). Although the mechanisms related to activation/upregulation of these mCRP in USC are currently poorly understood, it is of interest that CD59 activation has been previously reported to be a result of increased acetylation of p53 (Donev *et al*, 2006), an oncosuppressor gene known to represent one of the most commonly overexpressed/mutated gene in USC (Kohler *et al*, 1992). Whatever the mechanism, the common

upregulation of mCRP in USC suggests that high expression of CD46, CD55 or CD59 may represent an additional molecular evasion strategy for these biologically aggressive endometrial tumours during the interaction with the host immune system.

Consistent with this hypothesis, in an attempt to understand the implication of downregulating CD46, CD55 and CD59 in USC overexpressing Her2/neu during trastuzumab-induced cytotoxicity, in this study, we have compared trastuzumab CDC in the presence or absence of siRNA specifically targeting each of these mCRP in bioassays. Although the baseline level of CDC with or without trastuzumab was low against USC cell lines, a significant increase in trastuzumab-mediated CDC was consistently detected after downregulation of CD55 and CD59 by siRNA in USC cell lines. In contrast, no significant increase in CDC was detected after downregulation of CD46 by siRNA in any of the USC cell lines studied. These data are in agreement with a number of previous studies showing an innate high resistance of USC to complement activity *in vivo* and *in vitro* (El-Sahwi *et al*, 2010), as well as previous reports showing that in classical pathway activation, CD55 and CD59, but not CD46, are most effective in preventing cell lysis (Gorter and Meri, 1999). Importantly, our current findings provide for the first time evidence that the high resistance to complement of these biologically aggressive endometrial tumours is at least in part related to overexpression of CD55 and CD59.

Next, primary USC cell lines overexpressing Her2/neu were tested in trastuzumab ADCC experiments in the presence of siRNA against CD46, CD55 and CD59 and PBL as source on NK cells but in the absence of complement. We found a significant increase in trastuzumab-mediated ADCC after downregulation of CD55 and CD59 by siRNA in USC cell lines. In contrast, no significant effect was noted after downregulation of CD46 by siRNA. Although surprising, these results obtained in the absence of serum as source of complement are in agreement with those of other groups who have previously suggested additional novel functions for CD55 and CD59 in tumour immune surveillance that may be unrelated to complement inhibition (Finberg *et al*, 1992; Caragine *et al*, 2002; Kusama *et al*, 2003; Macor *et al*, 2007). Indeed, blocking of CD55 and CD59 by antibodies and/or miniantibodies was recently reported to increase not only complement but also PBL-mediated killing of human lymphoma cells triggered by rituximab (Macor *et al*, 2007). Similarly, Finberg *et al* (1992) found that in the absence of serum, CD55 expression on target cells significantly inhibited cytotoxicity by human NK cells. Although not specifically tested in our experiments, it is possible, as previously documented by others (Finberg *et al*, 1992), that in the absence of serum, NK cells used in the assays may release soluble C3 in the culture medium and thereby influence the cytolytic process. Whatever the mechanism, these data combined with our results suggest that the high levels of expression of CD55 and CD59 mCRP found in USC may not only decrease CDC but also, affecting trastuzumab ADCC, function as a negative regulator of cellular immunity *in vitro* and likely *in vivo*.

Her2/neu overexpression and *c-erbB2* gene amplification are detected in a significant number of USC patients (Díaz-Montes *et al*, 2006; Grushko *et al*, 2008) and found to be associated with more aggressive biological behaviour and poor prognosis (Santin *et al*, 2005a, c; Morrison *et al*, 2006). These findings have led to consideration of Her2/neu as a potential marker for trastuzumab-based treatment in patients harbouring USC refractory to the standard adjuvant treatment (Villella *et al*, 2006; Santin *et al*, 2008). Consistent with this view, a prospective randomized clinical trial comparing the efficacy of trastuzumab in combination with carboplatin and paclitaxel vs carboplatin and paclitaxel alone in advanced/recurrent USC patients overexpressing Her2/neu is currently recruiting patients in multiple institutions in the USA (clinicaltrials.gov/ct2/show/NCT01367002). Taken together, the results of our current study suggest that the simultaneous attack of HER2/neu-overexpressing USC with trastuzumab and with

siRNA able to specifically target CD55 and CD59 *in vivo* might improve both CDC and trastuzumab ADCC against these biologically aggressive tumours. In this regard, although the lack of targeted siRNA delivery currently remains a major obstacle for its broad therapeutic *in vivo* application, recent evidence suggests that specific targeting of mCRP by nanoparticles and/or herceptin-conjugated liposomes is feasible and potentially able to improve antibody-based cancer immunotherapy (Aigner, 2007; Li et al, 2007, 2009).

In conclusion, this is the first report demonstrating that biologically aggressive USC express high levels of the mCRPs CD46, CD55 and CD59 and that siRNA inhibition of CD55 and CD59 is able to sensitise USC to both CDC and ADCC *in vitro*. Although *in vivo* data will ultimately be necessary to validate the therapeutic potential of trastuzumab in combination with inhibition of CD55 and CD59, our *in vitro* results strongly suggest that

siRNA inhibition of these mCRP, if specifically targeted to tumour cells, may represent an attractive and novel strategy to treat USC overexpressing HER2/neu refractory or resistant to chemotherapy.

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Conflict of interest

The authors declare no conflict of interest.

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