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FULL PAPER

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EpCAM expression varies significantly and is differentially associated with prognosis in the luminal B HER2^{+}, basal-like, and HER2 intrinsic subtypes of breast cancer

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Background: Epithelial cell adhesion molecule (EpCAM) is frequently expressed in breast cancer, and its expression has been associated with poor prognosis. Breast cancer can be subdivided into intrinsic subtypes, differing in prognosis and response to therapy.

Methods: To investigate the association between EpCAM expression and prognosis in the intrinsic subtypes of breast cancer, we performed immunohistochemical studies on a tissue microarray encompassing a total of 1365 breast cancers with detailed clinicopathological annotation and outcomes data.

Results: We observed EpCAM expression in 660 out of 1365 (48%) cases. EpCAM expression varied significantly in the different intrinsic subtypes. In univariate analyses of all cases, EpCAM expression was associated with a significantly worse overall survival. In the intrinsic subtypes, EpCAM expression was associated with an unfavourable prognosis in the basal-like and luminal B HER2^{+} subtypes but associated with a favourable prognosis in the HER2 subtype. Consistently, specific ablation of EpCAM resulted in increased cell viability in the breast cancer cell line SKBR3 (ER^{-}, PR^{-}, and HER2^{+}) but decreased viability in the breast cancer cell line MDA-MB-231 (ER^{-}, PR^{-}, and HER2^{-}).

Conclusion: The differential association of EpCAM expression with prognosis in intrinsic subtypes has important implications for the development of EpCAM-targeted therapies in breast cancer.

The epithelial cell adhesion molecule (EpCAM, also designated TACSTD1, CD326) is a type I transmembrane protein of 314 amino acids that is localised to the basolateral membrane in the majority of normal epithelial tissues. The functional role of EpCAM in cell adhesion was the focus of early studies, and EpCAM has been demonstrated to be a calcium-independent homophilic cell adhesion molecule (Litvinov *et al*, 1994). Recent studies have also demonstrated a role for EpCAM in cell signalling and carcinogenesis (Munz *et al*, 2004; Osta *et al*, 2004; Maetzel *et al*, 2009). EpCAM is perhaps best known for the fact that it is

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expressed in the majority of human epithelial cancers, including colorectal, breast, gastric, prostate, ovarian, and lung cancer (Spizzo *et al*, 2004; Went *et al*, 2004). EpCAM was the first human tumour-associated antigen to be identified with monoclonal antibodies (Herlyn *et al*, 1979) and was the first target of monoclonal antibody therapy in humans (Sears *et al*, 1982). EpCAM expression has also been used to enrich circulating tumour cells before microscopic evaluation in the only FDA-approved assay for detection of circulating tumour cells in breast cancer (Cristofanilli *et al*, 2004). Finally, EpCAM-targeted therapies remain under active investigation, and a recent report highlighted at least seven different molecular therapies targeting EpCAM in various cancer types, including breast, gastric, ovarian, and lung cancer (Baeuerle and Gires, 2007). Particular effort is being made for breast cancer patients, where EpCAM expression appears to predict response to EpCAM-targeting antibodies (Schmidt *et al*, 2010; Schmidt *et al*, 2012).

EpCAM expression in primary cancer specimens has been associated with a favourable prognosis in some cancer types and an unfavourable prognosis in other cancer types. For instance, EpCAM expression in primary breast and ovarian cancers as measured by immunohistochemistry is associated with poor overall survival (OS; Spizzo *et al*, 2002; Spizzo *et al*, 2004; Spizzo *et al*, 2006; Schmidt *et al*, 2008), while EpCAM expression in colorectal and gastric cancer is associated with more favourable prognosis (Songun *et al*, 2005; Went *et al*, 2006). All of these studies were performed using standardised assay conditions and several used tissue microarrays (TMAs); taken together, these studies include specimens from over 6000 cancer patients. This differential association between EpCAM expression and prognosis is paralleled in functional studies of EpCAM biology performed both *in vitro* and *in vivo*. Loss-of-function analyses using RNA interference suggest that EpCAM expression is associated with increased invasion in breast cancer (Osta *et al*, 2004; Sankpal *et al*, 2009a, b, 2011), and gain-of-function analyses in colorectal and lung cancer suggest that EpCAM expression is associated with decreased cancer invasion (Basak *et al*, 1998; Tai *et al*, 2007). Moreover, in breast cancer cell lines, the impact of EpCAM expression on proliferation has been recently shown to depend on the epithelial and mesenchymal phenotype of these cells (Martowicz *et al*, 2012). Taken together, these studies suggest that the impact of EpCAM expression on cancer biology may be context-dependent (reviewed in van der Gun *et al*, 2010).

Breast cancer is a heterogeneous disease. Recent advances in molecular biology, including the development of sophisticated techniques for gene expression profiling, have established a new taxonomy of breast cancer, defining the breast cancer intrinsic subtypes (Perou *et al*, 2000). This new taxonomy has had a profound impact on the clinical management of breast cancer, as the intrinsic subtypes differ markedly in prognosis and response to therapy. As gene expression profiling data are not always available, a recent international consensus conference defined an approximation, using immunohistochemical expression of oestrogen receptor (ER) and progesterone receptor (PR), overexpression, and/or amplification of the human epidermal growth factor receptor 2 (HER2) and Ki67 labelling index to approximate the breast cancer intrinsic subtypes (Goldhirsch *et al*, 2011).

Although EpCAM expression is associated with poor prognosis in breast cancer, the impact of EpCAM expression on prognosis in the breast cancer intrinsic subtypes remains to be defined. As noted above, this is an important question, as the breast cancer intrinsic subtypes have distinct biology, and there is strong evidence suggesting that the impact of EpCAM in cancer is context-dependent. To definitively address this question, we performed immunohistochemical studies on a breast cancer TMA encompassing a total of 1365 breast cancer cases with detailed clinical annotation and outcomes data. A better understanding of the relationship between EpCAM expression and prognosis in the breast cancer intrinsic subtypes has important implications for the design and successful application of molecular therapies targeting EpCAM in breast cancer.

Table 1. Basic demographic data for 1365 evaluable breast cancer cases

Mean tumour size (mm)	31.0	
Mean age at diagnosis (years)	63.5	
Tumour stage		
	Number (*n*)	**%**
pT1	364	26.7
pT2	728	53.4
pT3	104	7.6
pT4	169	12.3
Lymph node involvement		
pN0	701	51.5
pN1	529	38.8
pN2	132	9.7
Tumour grade		
1	318	23.3
2	551	40.4
3	496	36.3
Histological subtype		
Invasive ductal	977	71.6
Invasive lobular	187	13.7
Mucinous	38	2.8
Apocrine	17	1.2
Cribriform	41	3.0
Papillary	18	1.3
Medullary	43	3.2
Other	43	3.2
Intrinsic subtype		
Luminal A (ER^+ and/or PR^+, $HER2^-$, Ki-67 < 14%)	213	15.6
Luminal B (HER2-negative) ER^+ and/or PR^+, $HER2^-$, Ki-67 ⩾ 14%)	673	49.3
Luminal B (HER2-positive) (ER^+ and/or PR^+, $HER2^+$)	154	11.3
HER2 type (ER^- or PR^-, $HER2^+$)	111	8.2
Basal-like (ER^-, PR^-, $HER2^-$)	213	15.6

Abbreviations: ER = oestrogen receptor; HER2 = human epidermal growth factor receptor 2; pr = progesterone receptor.

MATERIALS AND METHODS

Tissue microarray. We used a TMA encompassing a total of 2020 breast cancer tissue punches from 1579 formalin-fixed and paraffin-embedded tumour samples. These samples were collected from patients diagnosed with primary breast cancer between 1985 and 2007 at the University of Basel and the Viollier Institute in Basel, Switzerland. Of these 2020 tissue punches, a total of 1365 cases were evaluable for our study. The tissue samples were

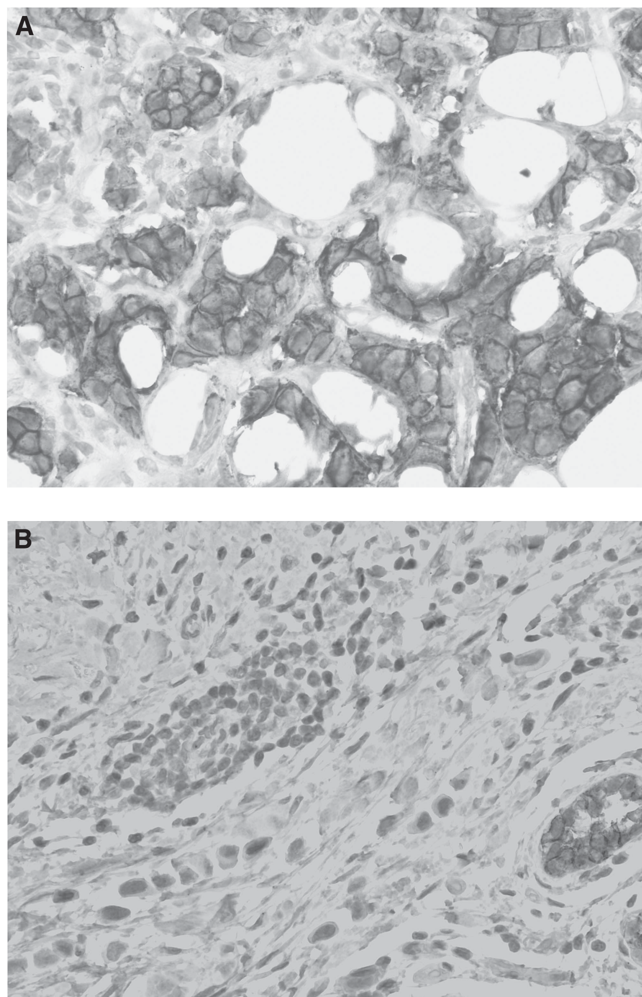

Figure 1. Representative photographs of EpCAM expression in breast cancer. (**A**) Strong membranous expression in 100% of tumour cells. (**B**) Negative expression on tumour cells with positive expression in normal mammary glands as an internal positive control. Original magnification ×400. A full color version of this figure is available at the *British Journal of Cancer* online.

brought into a TMA format as previously described (Bubendorf *et al*, 2001). Briefly, 0.6-mm tissue cylinders were punched out of donor tumour tissue blocks and transferred into a recipient paraffin block using a semi-automated tissue arrayer. Each TMA contained a number of tumour punches ranging from 159 to 522. Histopathological data were obtained from the original pathology reports, and patient outcomes data were obtained from the Cancer Registry of Basel or from the patient's attending physician. Retrieval of tissue and clinical data was performed in accordance with the regulations of the local institutional review boards, with specific attention to ethical standards and patient confidentiality.

Immunohistochemistry. Immunohistochemical staining of the TMA was performed as described previously (Tapia *et al*, 2011). For EpCAM immunohistochemistry, 4 μm sections of the TMA blocks were incubated overnight with a primary anti-EpCAM monoclonal antibody (1:800; clone VU-1D9, Novocastra, Newcastle, UK) after heat-induced antigen retrieval. Standard ABC-technique (ABC-Elite-Kit, Vector Laboratories, Burlingham, CA, USA) was used for immunostaining, and diaminobenzidine was used as chromogen. Counterstaining was performed with hematoxylin solution. The staining intensity of ER, PR, and HER2 was scored as previously described (Tapia *et al*, 2011). EpCAM expression was quantified using the modified Histo-score (H-score) (McCarty *et al*, 1985), with a range of possible scores from 0–300. Frequency and staining intensity of EpCAM on tumour cells were analysed, and EpCAM expression was

Table 2. Association between EpCAM expression and clinicopathological parameters

Clinicopathological parameter	EpCAM-positive		EpCAM-negative		*P* value
Mean tumour size (mm)	34.2		27.8		<0.0001
Mean age age at diagnosis (years)	63.7		63.3		0.6220
Tumour stage					**<0.0001**
pT1	132	36.3	232	63.7	
pT2	366	50.3	362	49.7	
pT3	65	62.5	39	37.5	
pT4	97	57.4	72	42.6	
Lymph node involvement					**0.0023**
pN0	329	46.9	372	53.1	
pN1	248	46.9	281	53.1	
pN2	83	62.9	49	37.1	
Tumour grade					**<0.0001**
1	99	31.1	219	68.9	
2	234	42.5	317	57.5	
3	327	65.9	169	34.1	
Oestrogen receptor					**<0.0001**
ER^+	439	42.9	583	57.1	
ER^-	221	65.2	118	34.8	
HER2					**0.0298**
$HER2^+$	144	54.3	121	45.7	
$HER2^-$	516	46.9	584	53.1	
Ki67					**<0.0001**
$Ki67^+$	593	55.4	477	44.6	
$Ki67^-$	66	23.0	221	77.0	

Abbreviations: EpCAM = epithelial cell adhesion molecule; ER = oestrogen receptor; HER2 = human epidermal growth factor receptor 2; PR = progesterone receptor.

Table 3. Association between EpCAM expression and histological subtype

	EpCAM-positive		EpCAM-negative		*P*-value
Histological subtype	***n***	**%**	***n***	**%**	**<0.0001**
Invasive ductal	496	50.8	481	49.2	
Lobular	48	25.7	139	74.3	
Mucinous	20	52.6	18	47.4	
Apocrine	13	76.5	4	23.5	
Cribiform	12	29.3	29	70.7	
Papillary	12	66.7	6	33.3	
Medullary	36	83.7	7	16.3	
Other	23	53.5	20	46.5	

Abbreviation: EpCAM = epithelial cell adhesion molecule.

Table 4. Association between EpCAM expression and breast cancer intrinsic subtype

Intrinsic subtype	EpCAM-positive		EpCAM-negative		P-value
	n	%	n	%	< 0.0001
Luminal A (ER^+ and/or PR^+, $HER2^-$, Ki-67 < 14%)	48	22.5	165	77.5	
Luminal B (HER2-negative) (ER^+ and/or PR^+, $HER2^-$, Ki-67 ⩾ 14%)	332	49.3	341	50.7	
Luminal B (HER2-positive) (ER^+ and/or PR^+, $HER2^+$)	63	40.9	91	59.1	
HER2 type (ER^-, PR^-, $HER2^+$)	81	73.0	30	27.0	
Basal-like (ER^-, PR^-, $HER2^-$)	136	63.8	77	36.2	

Abbreviations: EpCAM = epithelial cell adhesion molecule; ER = oestrogen receptor; HER2 = human epidermal growth factor receptor 2; PR = progesterone receptor.

Table 5. Univariate analyses for all cases, and by intrinsic subtype, for the effect of EpCAM expression on overall survival

EpCAM expression, all cases	Hazard ratio (95% CI)	P-value
EpCAM-positive	1.402 (1.178–1.668)	0.0001
EpCAM expression, by intrinsic subtype		
Luminal A	1.487 (0.817–2.707)	0.1937
Luminal B ($HER2^-$)	1.208 (0.936–1.560)	0.1464
Luminal B ($HER2^+$)	2.400 (1.451–3.971)	0.0006
HER2 type	0.374 (0.217–0.644)	0.0004
Basal-like	1.634 (1.070–2.497)	0.0231

Abbreviations: EpCAM = epithelial cell adhesion molecule; CI = confidence interval; HER2 = human epidermal growth factor receptor 2.

dichotomised into two groups according to the frequency distributions of the H-scores, using a cut-off score of ⩾100 (H-score 0–99 = negative/low expression and 100–300 = positive expression).

Statistical analysis. The distribution of patient and clinical characteristics between EpCAM-positive and EpCAM-negative tumours was compared using the Chi-square test, Wilcoxon's rank sum test, or two-sample *t*-test, as appropriate. OS was defined as the time from the first operation to death due to any cause. Survivors were censored at the date of last contact. Survival curves by EpCAM status were estimated using the Kaplan–Meier product-limit method and compared by log-rank test. Univariate Cox proportional hazard models were fit to identify factors significantly related to OS. To assess whether EpCAM was an independent predictor of survival, a multivariate Cox model was constructed to adjust other patient/clinical characteristics that were significant in the univariate analyses. Two-way interaction terms between EpCAM and other factors in the multivariate Cox model were also assessed. All analyses were two-sided and significance was set at a *P*-value of 0.05. Statistical analyses were performed using SAS (SAS Institutes, Cary, NC, USA).

Cell culture. All breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). MCF-10A cells were grown in DMEM/F12 medium supplemented with 5% donor horse serum, 20 ng ml^{-1} epidermal growth factor, 10 μg ml^{-1} insulin, 100 μg ml^{-1} hydrocortisone, and 1 ng ml^{-1} cholera toxin. MDA-MB-231 and SKBR3 cells were grown in DMEM medium supplemented with 10% FBS.

Antibodies and western blot analysis. Western blotting was performed by standard procedures. Cell lysates were harvested with RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1 × complete protease inhibitors (Roche, Basel, Switzerland), and phosphatase inhibitors, including sodium orthovanadate (Sigma, St. Louis, MO, USA) and sodium fluoride (Sigma). Protein concentration was measured using the BCA protein assay (Thermo Scientific, Rockford, IL, USA). Equal loading of each sample of protein (20 μg) was then subjected to SDS–PAGE (NuPAGE, Invitrogen, Carlsbad, CA, USA) and transferred by electrophoresis to a PVDF membrane. Antibodies against EpCAM C-10 (Santa Cruz, Dallas, TX, USA) and Actin C-4 (Santa Cruz) were used at ratios of 1 : 1000 and 1 : 5000, respectively, and detection was performed with HRP-conjugated antisera and chemiluminescence (Thermo Scientific, Rockford, IL, USA).

Lentiviral shRNA production and infections. Lentiviral vectors encoding shRNAs specific for *EpCAM* as well as control *GFP* sequences are part of The RNAi Consortium shRNA Library (http://www.broadinstitute.org/rnai/trc). Sequences are as follows: for shRNA EpCAM, 5′GCAAATGGACACAAATTACAA3′; and for shRNA GFP, 5′ACAACAGCCACAACGTCTATA3′. Lentivirus was produced by transfection of 293T cells with vectors encoding gene-specific shRNAs (1 μg) together with the packaging plasmids encoding Δ8.9 and VSV-G using Fugene 6 (Roche). Culture supernatants containing lentivirus were collected 48 and 72 h post transfection. Virus was pooled and stored at −80 °C. Cells were infected using a 1 : 11 dilution of virus in polybrene-containing media. Following centrifugation at 1000 **g** for 15 min, all breast cancer cell lines were selected in puromycin (1 μg ml^{-1}) starting 24 h post infection. Viability assays were conducted 10 days post infection using crystal violet. Protein lysates were harvested 72 h following shRNA expression to evaluate gene suppression.

Crystal violet cell proliferation assay. Cell proliferation assays were performed in six-well plates. Cells were plated to achieve 50% cell density on day of lentiviral infection. Puromycin selection 24 h post infection was performed. On day 3 post infection, cells were re-plated onto 60-mm dishes. On day 10, plates were stained with crystal violet (crystal violet 0.2%, ethanol 2%) and photographed. The crystal violet stain was then solubilised in 1% SDS solution with incubation on a shaker for 1 h at room temperature. Optical density was measured at 595 nm. Cell proliferation effects for SK-BR-3 after exposure to EpCAM shRNA and GFP shRNA were analysed as percentages. The solubilised dye from the EpCAM shRNA sample was read at 100% and then serially diluted to 75%, 50%, and 25% to generate a crystal violet curve to extrapolate the percentage for the GFP shRNA sample. Data presented are representative of three independent experiments.

RESULTS

The expression of EpCAM could be evaluated in 1365 cases in this cohort. Basic demographic information for the 1365 evaluable cases is presented in Table 1. The mean age at diagnosis was 63.5 years (range 27–101 years), and the mean follow-up time was 80.8 months (range 1–263 months). Expression of EpCAM was confined to the membrane of breast cancer cells in all the cases (Figure 1). EpCAM expression was defined using the H-score, a score integrating the intensity of EpCAM staining, and the percentage of breast cancer cells expressing EpCAM. With an H-score threshold of ⩾100, a total of 660 breast cancers

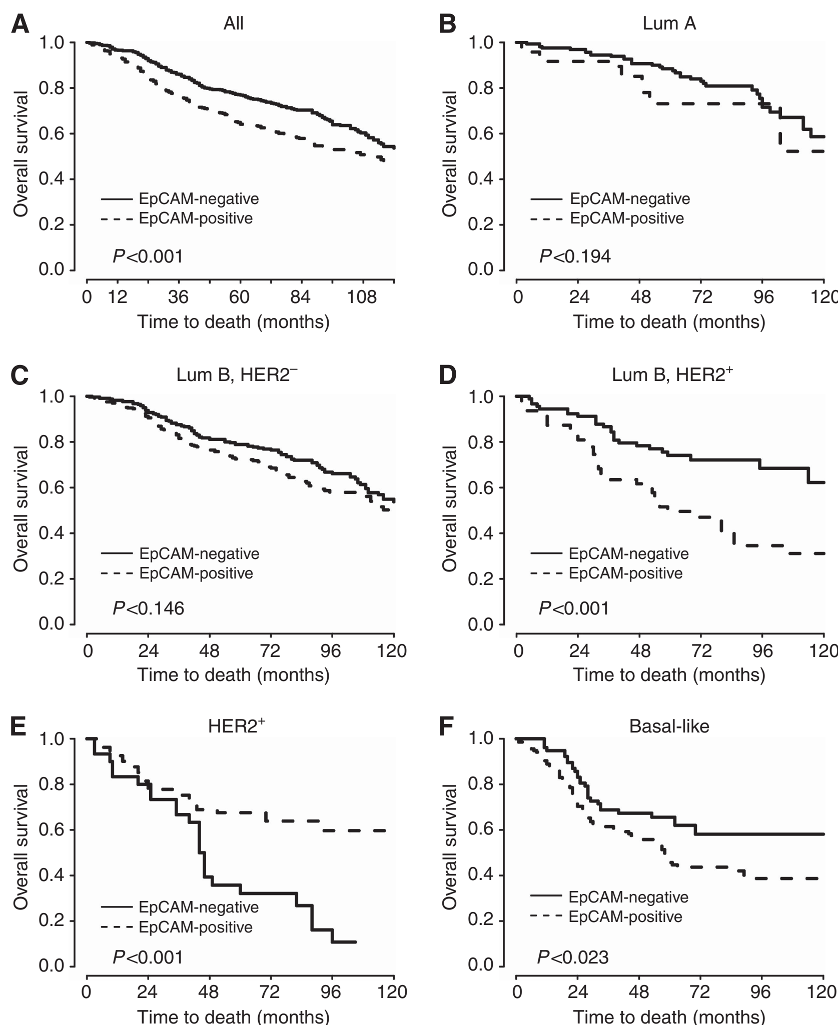

Figure 2. Kaplan–Meier survival curves. (**A**) Kaplan–Meier survival curve for overall survival depending on EpCAM overexpression (univariate analysis). (**B**–**F**) Kaplan–Meier survival curves for overall survival depending on EpCAM overexpression for indivdual intrinsic breast cancer subtypes.

(48.3%) expressed EpCAM. Consistent with previous studies, EpCAM expression was significantly associated with greater tumour size, lymph node involvement, tumour stage, and tumour grade (Table 2; Gastl *et al*, 2000; Schmidt *et al*, 2008; Agboola *et al*, 2011; Spizzo *et al*, 2011). EpCAM expression was significantly higher in ER-negative (ER^-) cases (EpCAM expression 65.1% in ER^- cancers *vs* 42.9% in ER^+ cancers, $P<0.0001$, Table 2) and in $HER2^+$ cases (EpCAM expression 54.3% in $HER2^+$ cancers *vs* 46.9% in $HER2^-$ cancers, $P=0.0298$, Table 2). EpCAM expression was also significantly associated with histological subtype ($P<0.0001$); EpCAM expression was higher in the invasive ductal (51%) and medullary (84%) histological types and lower in the invasive lobular (26%) and cribiform (29%) subtypes (Table 3). EpCAM expression varied significantly between the intrinsic subtypes of breast cancer, as defined by the St. Gallen Consensus Conference criteria (Goldhirsch *et al*, 2011). EpCAM overexpression was highest in the basal-like (64%) and HER2 (73%) subtypes and lowest in the luminal A subtype (23%, $P<0.0001$; Table 4). Furthermore, EpCAM expression was highly correlated with the expression of Ki-67 in all of the intrinsic subtypes except for luminal A (data not shown).

In univariate survival analyses, we could demonstrate that breast cancer cases with EpCAM expression had a significantly worse OS (hazard ratio (HR) $=1.402$, $P=0.0001$; Table 5 and Figure 2). In univariate analyses of the breast cancer intrinsic subtypes, EpCAM expression was associated with significantly decreased OS in the luminal B $HER2^+$ subtype ($HR=2.4$, $P=0.0006$) and the basal-like subtype ($HR=1.634$, $P=0.023$; Table 5 and Figure 2). The luminal A and the luminal B $HER2^-$ subtypes also showed a trend towards decreased OS with EpCAM expression (Table 5 and Figure 2), but this was not statistically significant. Of particular interest, EpCAM expression was significantly associated with improved OS in the HER2 subtype ($HR=0.374$, $P=0.0004$; Table 5 and Figure 2). In multivariate analysis, after adjusting for age, tumour size, lymph node involvement, tumour grade, and intrinsic subtype, EpCAM expression remained significantly associated with improved OS in the HER2 subtype ($HR=0.329$, $P<0.0001$) and showed a trend toward decreased OS in the luminal B $HER2^+$ subtype ($HR=1.546$, $P=0.094$) and the basal-like subtype ($HR=1.437$, $P=0.096$; Table 6).

Previously, we demonstrated that specific ablation of EpCAM significantly decreased proliferation of the basal-like breast cancer cell line MDA-MB-231, with minimal impact on proliferation of the breast epithelial cell line MCF10A. Given the differential association between EpCAM and prognosis in the HER2 and basal-like intrinsic subtypes of breast cancer, we sought to evaluate the impact of EpCAM expression on proliferation in representative breast cancer cell lines *in vitro*. We specifically ablated EpCAM expression in the MCF10A, MDA-MB-231, and SKBR3 (ER^-/PR^-/$HER2^+$) cell lines (Figure 3). Of note, we not only confirmed

Table 6. Multivariate analysis for the effect of clinicopathological parameters and EpCAM expression on overall survival

Clinicopathological parameter	Hazard ratio (95% CI)	*P*-value
Age (per 1 year)	1.037 (1.029–1.044)	<0.0001
Tumour stage		
pT1 (reference)	1	
pT2	1.603 (1.226–2.097)	0.0006
pT3	2.089 (1.437–3.037)	0.0001
pT4	2.208 (1.581–3.083)	<0.0001
Lymph node involvement		
pN1 (reference)	1	
pN1	1.427 (1.168–1.745)	0.0005
pN2	2.782 (2.103–3.680)	<0.0001
Tumour grade		
BRE grade 1 (reference)	1	
Grade 2	1.631 (1.230–3.454)	0.0006
Grade 3	2.571 (1.914–3.454)	<0.0001
Intrinsic subtype		
Luminal A (reference)	1	
Luminal B (HER2$^-$)	1.081 (0.792–1.475)	0.6237
Luminal B (HER2$^+$)	1.263 (0.864–1.848)	0.2283
HER2 type	1.193 (0.784–1.815)	0.4097
Basal-like	2.031 (1.419–2.906)	0.0001
EpCAM expression, by intrinsic subtype		
Luminal A	1.404 (0.771–2.557)	0.2666
Luminal B (HER2$^-$)	0.860 (0.660–1.119)	0.2614
Luminal B (HER2$^+$)	1.546 (0.928–2.575)	0.0941
HER2 type	0.329 (0.190–0.570)	<0.0001
Basal-like	1.437 (0.938–2.201)	0.0958

Abbreviations: EpCAM = epithelial cell adhesion molecule; BRE = Elsten's modification of Bloom and Richardson; CI = confidence interval; HER2 = human epidermal growth factor receptor 2.

that specific ablation of EpCAM decreases proliferation in the MDA-MB-231 cell line but also demonstrated that specific ablation of EpCAM results in an almost four-fold increase in proliferation in the SKBR3 cell line, consistent with the IHC analyses.

DISCUSSION

EpCAM is commonly expressed in breast cancer, and EpCAM expression in primary breast cancers has been widely reported to be associated with poor prognosis. However, breast cancer is a heterogeneous disease, and the impact of EpCAM expression on prognosis in the breast cancer intrinsic subtypes remains to be investigated. Here, we studied EpCAM expression in a large cohort of primary breast cancers, with a particular focus on defining the potential associations between EpCAM expression and the breast cancer intrinsic subtypes, as defined by the most recent St. Gallen Consensus Conference (Goldhirsch *et al*, 2011). Of note, we demonstrate for the first time that EpCAM expression varies significantly between the intrinsic subtypes of breast cancer, ranging from 22.5% in luminal A to 73.0% in the HER2 subtype. We have also made the surprising observation that the impact of EpCAM expression on prognosis in breast cancer is dependent on intrinsic subtype. In the basal-like and luminal B HER2$^+$ subtypes, EpCAM expression is associated with an unfavourable prognosis; in the HER2 subtype, EpCAM expression is associated with a favourable prognosis.

Our study confirms the results of previous studies demonstrating that EpCAM expression is associated with tumour size, lymph node involvement, tumour grade, and prognosis in breast cancer (Tandon *et al*, 1990; Schmidt *et al*, 2008; Spizzo *et al*, 2011; Agboola *et al*, 2011). In addition, we observed that EpCAM expression is inversely associated with expression of ER ($P < 0.0001$) and positively associated with HER2 expression ($P = 0.0298$), findings that have been previously reported by others (Spizzo *et al*, 2002; Schmidt *et al*, 2008; Agboola *et al*, 2011). Although multiple studies have suggested that EpCAM expression is an independent predictor of disease-free survival and OS (Spizzo *et al*, 2002; Schmidt *et al*, 2008; Agboola *et al*, 2011), we did not observe this finding in our collective ($P = 0.7824$ in multivariate analysis). This may be related to the strong association we observed between EpCAM expression and traditional predictors of prognosis in breast cancer, such as tumour size, lymph node involvement, tumour grade, ER status, HER2 status, intrinsic subtype, and Ki67 labelling index.

This study is the first to evaluate the association between EpCAM expression and prognosis in the intrinsic subtypes of breast cancer. To do this, we used well-recognised criteria for approximating the intrinsic subtypes using immunohistochemical criteria (ER status, PR status, HER2, Ki67 labelling index). Because of the large sample size ($n = 1365$ cases), we had adequate power to meaningfully evaluate potential associations between EpCAM expression and prognosis in the breast cancer intrinsic subtypes, demonstrating a surprising differential impact on prognosis between the basal-like, luminal B HER2$^+$, and HER2 subtypes. Recently, Agboola *et al* (2011) investigated the impact of EpCAM expression on prognosis in a cohort of 726 primary breast cancer cases. They observed that EpCAM expression is associated with tumour size and tumour grade and is an independent predictor of disease-free and OS. They also evaluated the potential association between EpCAM expression and other biomarkers, including ER, HER2, p53, CK5/6, and CK14. Of note, they defined basal-like breast cancer as CK5/6 and/or CK14-positive and demonstrated that EpCAM expression is an independent predictor of poor prognosis in basal-like breast cancer. Although Agboola *et al* (2011) use a different criteria to define basal-like breast cancer, their results are consistent with the results reported here, confirming the importance of EpCAM expression in this intrinsic subtype. EpCAM-targeted therapies may be particularly appropriate in this subtype, because EpCAM is expressed in the majority of basal-like breast cancers and treatment options are otherwise limited for patients with basal-like breast cancer.

One limitation of our study is that we defined breast cancer intrinsic subtype using the St. Gallen Consensus Conference criteria. These criteria provide only an approximation of intrinsic subtype. For instance, not all triple-negative breast cancers identified by immunohistochemistry correspond to the intrinsic basal-like subtype. Although there is an approximately 80% overlap between triple-negative and intrinsic basal-like subtype, triple-negative cancers also include special histological subtypes, such as medullary and adenoid cystic carcinoma with a much lower risk of recurrence (Goldhirsch *et al*, 2011; Penault-Llorca and Viale, 2012). Thus, using the triple-negative phenotype as an approximation for basal-like breast cancer may underestimate the impact of EpCAM on this intrinsic subtype.

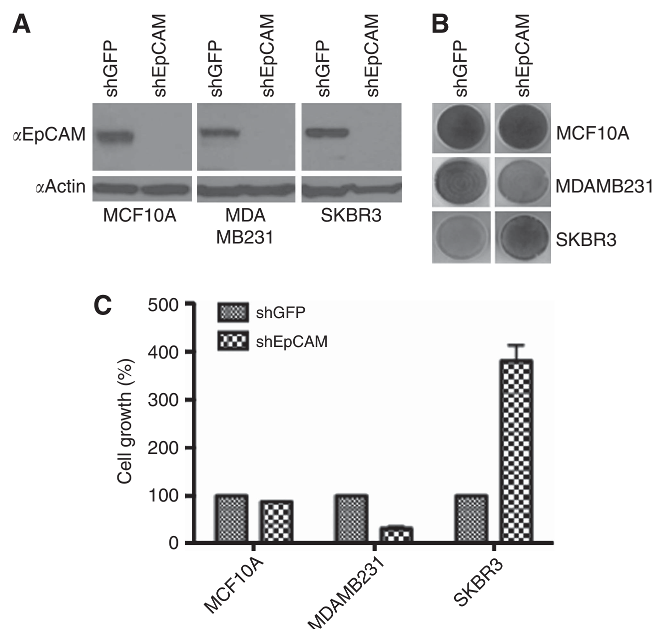

Figure 3. Specific ablation of EpCAM results in increased SKBR3 breast cancer cell viability. (**A**) EpCAM-shRNA specifically ablates EpCAM protein levels in MCF-10A, MDA-MB-231, and SKBR3 cells 72 h post infection relative to shGFP control vector. (**B**) Crystal violet-stained plates depicting cell growth differences in MCF10A, MDA-MB-231, and SKBR3 cells 14 days post infection. Plates representative of three independent experiments. (**C**) Crystal violet growth assay used to quantify the growth differences between the breast cancer cell lines. A full color version of this figure is available at the *British Journal of Cancer* online.

In this study, we demonstrate, for the first time, that EpCAM is highly expressed in the HER2 subtype (73.0%). Surprisingly, EpCAM expression in the HER2 subtype was associated with a significantly improved OS ($P=0.0004$). Consistent with these findings were the results of *in vitro* studies, where downregulation of EpCAM with shRNA led to increased viability and cell growth in the SKBR3 breast cancer cell line. This cell line corresponds to the HER2 subtype (ER$^-$ and PR$^-$, HER2$^+$), and its increased viability after specific ablation of EpCAM supports our finding that EpCAM is associated with favourable prognosis in this breast cancer subtype. The reason for this differential association with survival in the HER2 subtype is not known to date. Of note, a study by Spizzo *et al* (2002) suggests that concurrent EpCAM and HER2 expression may be associated with an additive negative impact on disease-related OS. In their study, however, the HER2$^+$ cases were evaluated as a single group. The current taxonomy of breast cancer suggests that HER2$^+$ cases should be divided into two subtypes, the luminal B HER2$^+$ subtype (ER$^+$ and/or PR$^+$, HER2$^+$) and the HER2 subtype (ER$^-$ and PR$^-$, HER2$^+$). In addition, Spizzo *et al* (2002) observed simultaneous expression of HER2 and EpCAM in a much smaller number of cases (13.2% *vs* 54.3% in our study). However, our study contains significantly more patient samples, and for this reason, our observations might have more power in this regard. Our data clearly suggest that EpCAM expression in the HER2 subtype is associated with a favourable prognosis. Therefore, targeting EpCAM in this group of patients should be performed with caution, particularly if the therapy is aimed at abrogating EpCAM-dependent signalling pathways.

A second limitation of this study is that we evaluated expression of extracellular EpCAM only. Recent evidence suggests that EpCAM can be cleaved at the cell surface, with nuclear translocation of the intracellular portion (Maetzel *et al*, 2009). Although we believe that expression of extracellular EpCAM is a surrogate for EpCAM-dependent signalling, staining with an antibody to the intracellular portion may provide better insight into the extent of EpCAM-dependent signalling. Further studies with a particular emphasis on defining EpCAM signalling may provide additional insights into the relationship between EpCAM expression and breast cancer biology.

In summary, we demonstrate that EpCAM expression is variably expressed in the breast cancer intrinsic subtypes and is differentially associated with OS in the luminal B HER2$^+$, HER2, and basal-like intrinsic subtypes. This result is consistent with the hypothesis that the impact of EpCAM expression on cancer biology is context-dependent and has important implications for the rational development of novel therapeutics targeting EpCAM currently under clinical investigation.

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