Sex Hormone-regulated *CMG2* Is Involved in Breast and Prostate Cancer Progression

ZIQIAN FANG, CHARLOTTE KILLICK, CERITH HALFPENNY, NATASHA FREWER,
KATHRYN A. FREWER, FIONA RUGE, WEN G. JIANG and LIN YE

*Cardiff China Medical Research Collaborative, Division of Cancer and Genetics,
Cardiff University School of Medicine, Cardiff, U.K.*

Abstract. *Background/Aim: Capillary morphogenesis gene 2 (CMG2) is involved in prostate and breast cancer progression. This study aimed to investigate sex hormone receptor-mediated regulation of CMG2 in breast and prostate cancer, and its implication in disease progression. Materials and Methods: Expression of CMG2, oestrogen receptor (ER) and androgen receptor (AR) was determined in breast and prostate cancer cell lines, respectively, using real-time quantitative PCR (QPCR) and western blot. Association between CMG2 and sex hormone receptors was analysed in a number of transcriptome datasets. Immunochemical staining was performed in tissue microarrays of breast cancer (BR1505D) and prostate cancer (PR8011A). CMG2 expression was determined in 17β-oestradiol treated breast cancer cells and AR over-expressing prostate cancer cells. Results: CMG2 was found to be inversely correlated with sex hormone receptors in breast and prostate cancer. Lower expression of CMG2 was associated with a poor prognosis in ER (+) breast cancer but not ER (–) tumours. Both ER (+) breast cancer cell lines and AR (+) prostate cancer cell lines presented lower expression of CMG2, which was increased following sex hormone deprivation. Exposure to 17-β-oestradiol and AR over-expression repressed CMG2 expression in breast cancer and prostate cancer cell lines, respectively. Conclusion: CMG2 is inversely correlated with ER and AR status in breast and prostate cancer, respectively. ER and AR mediate repression of CMG2 expression in corresponding cancerous cells.*

Correspondence to: Dr. Lin Ye, Cardiff China Medical Research Collaborative, Division of Cancer and Genetics, Cardiff University School of Medicine, Academic Avenue, Cardiff, CF14 4XN, U.K. Tel: +44 2920687861, e-mail: YeL@Cardiff.ac.uk

Breast cancer is the most common type of cancer in the UK, accounting for 15% of all cancer cases. Biomarkers, such as the oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER2) hold great weight in breast cancer prognosis (1). ER expression determines the validity of therapeutic interventions and is the best indicator to a positive response with an endocrine therapy (2). Tamoxifen is only prescribed as a treatment in ER positive (ER+) breast cancer; it acts as a pro-drug that inhibits oestrogen-dependent cellular proliferation (3). Therefore, it is not effective as a treatment for ER negative (ER-) breast cancer.

Androgen receptor (AR) plays a pivotal role in the development of prostate and maintains its physiological function by transferring the signal from extracellular ligands to intracellular pathways (4). Deregulated AR has been implicated in prostate cancer and has been targeted by common therapies for both locally advanced lesions and the most advanced diseases either to reduce its ligands produced from testis and adrenal glands or interfere with AR using specific inhibitors such as enzalutamide (5). These therapies are effective for early-stage tumours. However, when prostate cancer cells adapt to androgen deprivation and restore AR signalling, castration-resistant prostate cancer (CRPC) develops (6), and furthermore, prolonged AR pathway inhibition could cause histological dedifferentiation, epithelial–mesenchymal transition (EMT) and neuroendocrine differentiation.

Capillary morphogenesis gene 2 (*CMG2*) also known as anthrax toxin receptor 2 (ANTXR2) encodes the type I transmembrane protein CMG2. Its extracellular domain mediates cell adhesion to extracellular matrix by binding to collagen IV laminin and fibronectin (7). *CMG2* is located on chromosome 4q, and encodes four different protein isoforms CMG2[489], CMG2[488], CMG2[386], and CMG2[322] (8). These isoforms include vWA, Ig like, transmembrane and cytoplasmic domains. However, the CMG2[322] isotype is anticipated to be a secreted form as it lacks the transmembrane domain (8, 9). *CMG2* mutations are associated with juvenile hyaline fibromatosis (JHF) and infantile systemic hyalinosis (ISH). The *CMG2* mutations lie in the domain that is involved in the

extracellular matrix interaction, resulting in characteristic fibromatoses as hyaline accumulation in the dermis (10). *CMG2* has been shown to be up-regulated in human tumour endothelium. Furthermore, CMG2 is thought to regulate endothelial cell proliferation and tubule formation (9, 11). In addition to its role in angiogenesis, our previous study of CMG2 in prostate cancer showed that CMG2 was able to enhance the adhesion and but inhibited invasiveness of prostate cancer cells (12). A study of serum levels of CMG2 in prostate cancer patients showed increased CMG2 levels in patients who developed distant metastases (13). A study of its role in breast cancer demonstrated that over-expression of CMG2 inhibited the growth of breast cancer cells. Furthermore, reduced expression of CMG2 in breast cancer was associated with shorter overall and relapse-free survival (14).

The progression and prognosis of both breast and prostate cancer are significantly associated with sex hormone receptors. Furthermore, based on our previous studies of CMG2 in both prostate and breast cancer, we aimed to investigate whether sex hormone receptors regulate CMG2 and whether deregulated CMG2 is implicated in the progression of both breast and prostate cancer with a different status of these hormone receptors.

Materials and Methods

Cell lines and cell culture. The human breast cancer cell lines MCF-7, MDA-MB-231, T47D, ZR751, BT474, MDA-MB-361, SKBR3, BT549, BT20, MDA-MB-468 and prostate cancer cell lines PC-3, DU-145, LNCaP, VCaP were purchased from ATCC (American type culture collection, Boulevard Manassas, VA, USA). LNCAP, VCAP, BT474, MDA-MB-361 cells were cultured RPMI 1640 medium (Sigma Aldrich, Poole, UK) with 10% foetal calf serum (FCS) and antibiotics, whilst other cell lines were cultured in DMEM-F12 medium (Sigma Aldrich) supplemented with 10% FCS and antibiotics. Polyclonal goat anti-human-CMG2 (AF2940) antibody was obtained from R&D systems (Minneapolis, MN, USA).

Immunohistochemical staining for CMG2 in both prostate and breast cancer tissues. Tissue microarrays of both prostate cancer (PR8011a) and breast cancer (BR1505d) were purchased from US Biomax, Inc (Rockville, MN, USA). Immunohistochemical staining for CMG2 was performed using the VECTASTAIN® ABC Systems (Vector Laboratories, Oxfordshire, UK) and anti-CMG2 antibody (1:50, Abcam, Cambridge, UK).

AR over-expression in prostate cancer cell line. PC-3 and DU-145 cells were transduced with lentiviral particles prepared from lentiviral vectors carrying the coding sequence of human AR gene or empty vector, respectively (Vector builder, Chicago, IL, USA). Lentiviral particles were packaged in HEK-293T cells (GenHunter, Nashville, TN, USA). The transduced cells were subject to a selection using puromycin (2 μg/ml) before they were subsequently maintained in medium containing 0.5 μg/ml of puromycin.

RNA extraction, reverse transcription, and real time quantitative PCR (QPCR). Total RNA was extracted from cells using TRI reagent (Sigma-Aldrich). GoScript reverse transcription mix (Promega, Southampton, UK) was used for the reverse transcription. QPCR was employed to determine *CMG2* transcripts using Sybr Green master mix (Sigma Aldrich) and primers for CMG2 (forward:5'-CAGGATAGGTGCAGGACAAAGC and reverse: 5'-TCGGAATGGCAGTGTTCTCTGC) and GAPDH (forward: 5'-TGCACCACCAACTGCTTAGC-3' and reverse: 5'-GGCATGGACTGTGGTCATGAG-3'). Fold Change was calculated using 2–ΔΔCT method (15). For conventional PCR, GoTaq Green Master Mix (Promega, Hampshire, UK) was applied using primers for AR (forward:5'-TTACACCAAAGGGCTAGAAG and reverse: 5'-AGGGTACCACACATCAGGT-3') and GAPDH (forward: 5'-CTGAGTACGTCGTGGAGTC and reverse: 5'-GACTGTGGTCATGAGTCCTT).

Western blot analysis. Cellular proteins were extracted with RIPA lysis buffer followed by a separation using SDS-PAGE before electrical transfer onto a nitrocellulose membrane. A blocking with 10% skimmed milk was conducted before incubating with a primary antibody and corresponding secondary antibody. Antibodies used were anti-GAPDH (1:4,000, Santa Cruz Biotechnology, Dallas, TX, USA), anti-AR (1:2,000, Santa Cruz Biotechnology), anti-CMG2 (1:2,000, R&D Systems) and anti-Actin antibodies (1:4,000, R&D Systems). Secondary antibodies used include anti-mouse IgG (A5278, Sigma Aldrich) and anti-goat IgG (A8919, Sigma Aldrich). Protein bands were visualised using the Supersignal™ West Dura kit (Pierce Biotechnology, Rockford, IL, USA) and photographed with an UVITech imager (UVITech, Cambridge, UK).

Treatment of cell lines with 17-β-oestradiol. To remove endogenous serum steroids and any weak oestrogen activity, MCF-7 and MDA-MB-231 cells were washed twice with PBS and were subsequently cultured in 6-well plates in phenol red-free medium supplemented with 10% charcoal stripped foetal bovine serum (CS-FBS). After 24 h, cells were treated with 1nM 17-β-oestradiol for 4 and 24 h.

Statistical analysis. Mann–Whitney test was applied for non-normally distributed data, *t*-test was used for normally distributed data and Pearson's Chi-squared test was applied for analysing nominal data. Correlation between genes was analysed using Spearman test. All the statistical analyses were performed using SPSS software (version 26, SPSS, Chicago, IL, USA). Statistical significance was considered at $p<0.05$. Survival analysis was conducted using the KMplot (16).

Results

CMG2 expression in breast cancer is correlated with ER. *CMG2* expression was first analysed in a GEO dataset of breast cancer (GSE20685, n=327). *CMG2* expression was lower in ER (+) breast tumours (n=204), $p<0.05$ compared with ER (–) tumours (n=123) (Figure 1A). In the same cohort, *CMG2* expression was inversely correlated with ER (Figure 1B). Our previous study showed that high CMG2 expression is associated with better survival (14). To further determine whether this association is correlated with ER status, TCGA datasets (n=1044) were applied. In ER (+) tumours, CMG2 tended to be reduced in locally advanced tumours according to the T stage
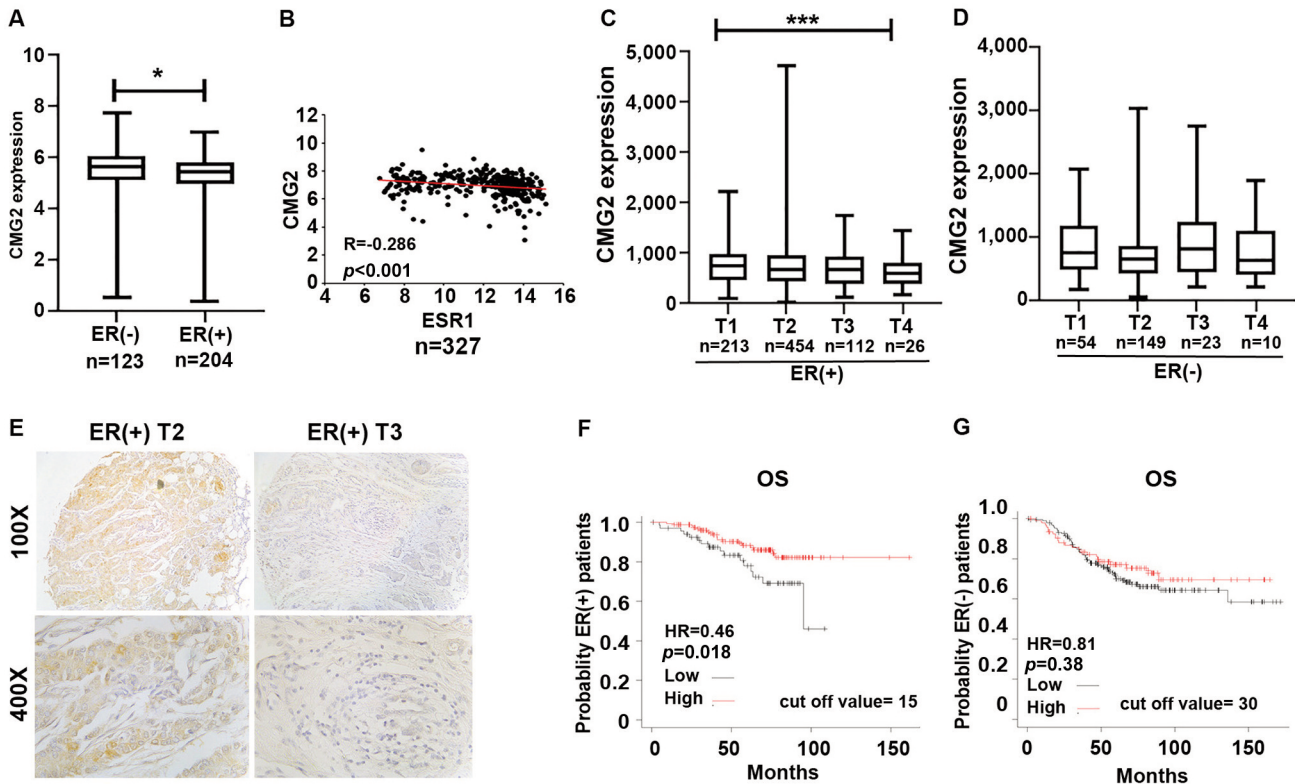
Figure 1. *Capillary morphogenesis gene 2 (CMG) is correlated with oestrogen receptor (ER) expression in breast cancer. (A) CMG2 transcript levels in ER (+) and ER (–) breast cancers were analysed in the GEO Dataset GSE20685 using Mann–Whitney test. *p<0.05. (B) Correlation between CMG2 and ESR1 (Erα) in a cohort of breast cancer (GSE20685) was analysed using Spearman test. In TCGA dataset (n=1,044), CMG2 expression levels were evaluated in both ER (+) tumours (C, n=805) and ER (–) tumours (D, n=236). (E) Representative images of the CMG2 immunohistochemistry (IHC) staining taken from ER(+) samples of breast cancer tumours at different T stages. Association with overall survival (OS) was analysed in both ER (+) tumours (F, n=221, cut off value=15) and ER (–) tumours (G, n=284, cut off value=30) using the KMplot (www.kmplot.com). *p<0.05, **p<0.01, ***p<0.001.*

(Figure 1C). However, such a change was not observed in the ER (–) tumours (Figure 1D), suggesting ER may play a role in regulating CMG2. Neither ER (+) nor ER (–) tumours exhibited differential expression of CMG2 according to lymph node metastasis and distant metastases (data not shown). To validate the CMG2 protein expression, immunohistochemical staining for CMG2 was performed in the tissue microarray of breast cancer (BR1505D). In ER (+) breast cancer tumours, CMG2 expression was decreased in tumours with a later T stage (T3 and T4) (Table I and Figure 1E). Furthermore, lower CMG2 expression was associated with poorer overall survival (OS) in patients with ER (+) tumours (Figure 1F) but not in those with ER (–) tumours (Figure 1G).

CMG2 expression is regulated by oestrogen in breast cancer cells. Expression of CMG2 in a panel of breast cancer cell lines was determined using both QPCR (Figure 2A) and western blot (Figure 2B). *CMG2* mRNA is highly expressed in three triple negative breast cancer (TNBC) cell lines (MDA-

MB-231, BT549 and BT20). Moderate expression was seen in a TNBC cell line (MDA-MB-468) and a HER2 positive cell line (HCC1419). CMG2 was lowly expressed or absent from ER (+) cell lines (MCF-7, ZR751, BT474 and MDA-MB-361) and another HER2 positive cell line (SKBR3). CMG2 protein was more abundant in MDA-MB-231 cells and moderately expressed in BT549 cells but absent or just detectable in other cell lines (Figure 2B). Expression of CMG2 transcripts in another panel of breast cancer cell lines was also evaluated in the CCLE dataset (Cancer Cell Line Encyclopaedia=44) (17). *CMG2* was inversely correlated with the ER expression in the breast cancer cell lines (Figure 2C). Exposure to 17-β-oestradiol for 24 h resulted in an approximately 50% reduction of *CMG2* transcripts in MCF-7 cells in comparison with its expression in the control and cells treated with 17-β-oestradiol for 4 h (Figure 2D). Furthermore, analysis of CMG2 expression in ERα-silenced MCF7 cells (GSE27473, CMG2: 238050_at) showed increased expression of *CMG2* compared with the control (Figure 2E).

Table I. *Capillary morphogenesis gene 2 (CMG2) immunohistochemistry (IHC) staining in breast tumours with different oestrogen receptor (ER) expression and different T stage.*

ER status	T stage	CMG2 stain strength				
		Negative	Weak	Moderate	Strong	*p*-Value
ER (–)	T1&T2	22 (41.5%)	16 (30.1%)	13 (24.5%)	2 (3.8%)	0.655
	T3&T4	20 (52.6%)	8 (21.1%)	7 (18.4%)	3 (7.9%)	
ER (+)	T1&T2	18 (42.9%)	12 (28.6%)	10 (23.8%)	2 (4.8%)	0.015
	T3&T4	14 (87.5%)	0 (0%)	2 (13.5%)	0 (0%)	

CMG2 is associated with prostate cancer progression especially for AR positive tumours. Thomas *et al.* found CMG2 transcript levels were up-regulated in metastatic prostate cancer compared with the primary tumour (13). To further validate this at the protein expression level, immunohistochemical staining for CMG2 was carried out in the tissue microarray of prostate cancer (PR8011A). Decreased staining for CMG2 was observed in the tumours in comparison with both normal prostate tissues and adjacent normal prostatic tissues (Figure 3A and B). Furthermore, our analysis of CMG2 expression in primary prostate tumours showed marked down-regulation of CMG2 in primary tumours (GSE3325) that developed distant metastases (n=6), *p*<0.05, compared with its expression in tumours without distant metastasis (n=13) (Figure 3C). Similarly, reduced expression of CMG2 was also observed in metastatic tumours (GSE6919) from prostate cancer in comparison with its expression in primary tumours (Figure 3D). The implication of CMG2 in the disease progression was also analysed in TCGA prostate cancer cohort according to different AR status. Analyses of its transcripts using RNA sequencing data showed that CMG2 was reduced in tumours that had lymph node metastasis but only in tumours with higher AR expression (Figure 3E). More interestingly, in tumours with higher AR expression, down-regulation of CMG2 was observed in tumours with a Gleason score greater than 7 (Figure 3F).

AR mediates a repression on CMG2 expression in AR positive prostate cancer cells. The down-regulation of CMG2 was observed in AR highly expressing prostate cancers that developed lymph node metastases and those with a higher Gleason score. Correlation between *CMG2* and *AR* was analysed in a gene array data of prostate cancer (GSE6919) comprising normal prostate tissues (n=17), adjacent normal prostate tissues (n=60), prostate primary tumours (n=66) and metastatic tumours (n=25). This analysis showed an inverse correlation between these two genes at the transcript level (Figure 4A). Quantitative analysis of *CMG2* transcripts showed that AR positive prostate cancer cell lines (LNCaP and VCaP) had lower expression of *CMG2* compared with the two AR negative prostate cancer cell lines (PC-3 and DU-145) (Figure 4B). PC-3 and DU-145 cell lines over-expressing AR, which was verified using conventional PCR (Figure 4C) and western blot (Figure 4D), exhibited reduced expression of CMG2 (Figure 4E). Furthermore, CMG2 expression was increased in the AR positive LNCaP cells when they were deprived from steroid hormones using charcoal stripped serum (Figure 4F).

Discussion

It has been shown that CMG2 is down-regulated in breast cancer and its reduced expression is associated with poor prognosis (14). To date, ER is one of the pivotal markers for characterising the disease, predicting prognosis, and treating the disease. In the present study, a decrease in CMG2 was evident in the more locally advanced ER (+) tumours but not in ER (–) breast cancers. This suggests that there is an association between ER and CMG2 in breast cancer and CMG2 is likely involved in the progression of the disease. Further analysis showed that CMG2 was inversely correlated with ERα. In line with the inverse correlation observed in breast cancer tumours, ER positive breast cancer cell lines also exhibited lower expression of CMG2 in comparison with the ER negative breast cancer cell lines, suggesting ER may mediate repression of CMG2 expression in breast cancer cells. Indeed, 17-β-oestradiol down-regulated CMG2 expression in MCF7 cells. This is also supported by the finding that ERα silencing in MCF7 cells resulted in increased expression of CMG2. However, in our previous study, an ERα agonist (PPT, Propyl pyrazole triol) increased CMG2 expression in MCF7 cells, which was prevented by a specific antagonist (MPP, methyl-piperidino-pyrazole) (14). The exact effect of these ER selective regulators on gene expression in comparison with natural oestrogens and their application in the treatment of the disease are yet to be fully investigated. In addition, the reduced CMG2 expression in ER positive breast cancers was also associated with poorer overall survival in patients with ER positive tumours. Our
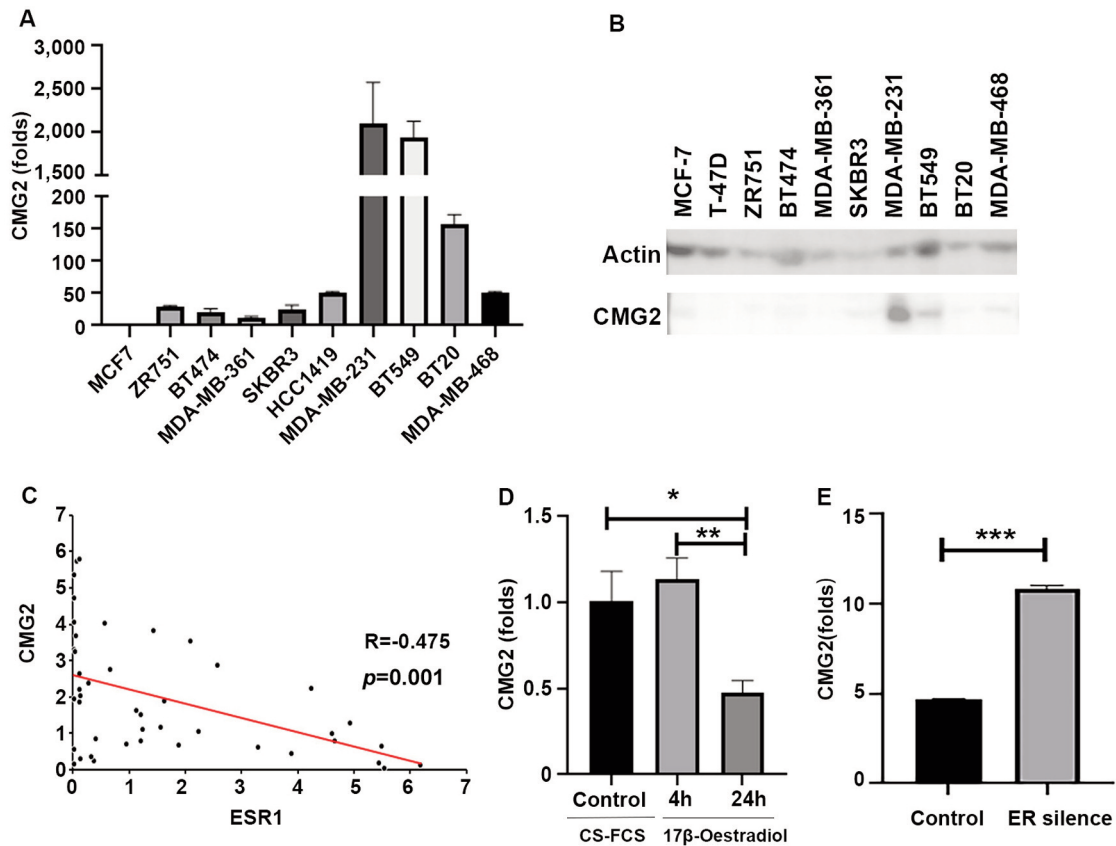
Figure 2. *Capillary morphogenesis gene 2 (CMG2) expression is regulated by oestrogen in breast cancer cells. (A) CMG2 transcripts were quantitatively analysed in breast cancer cell lines using QPCR. Fold change in gene expression calculated using the 2-ΔΔCT method. (B) CMG2 protein levels in the breast cancer cell lines were analysed using western blot. (C) Correlation between CMG2 and ESR1 (ERα) was analysed in breast cancer cell line data collected from the CCLE dataset using Spearman test. (D) CMG2 expression in MCF-7 cells treated with 17-β-oestradiol (1 nM) was quantified using QPCR. (E) CMG2 expression in Erα-silenced MCF-7 cells with (n=3) (GSE27473, CMG2: 238050_at) was also analysed in comparison with the control (n=3). *p<0.05, **p<0.01, ***p<0.001.*

previous study also showed that CMG2 over-expression can inhibit both proliferation and invasion of MCF7 cells (14). Taken together, the reduced CMG2 expression in ER positive breast cancer enhances proliferation and invasiveness of breast cancer cells and thus contributes to the disease progression and poor prognosis. Further investigation will shed light on the role of CMG2 in ER positive breast cancer and the corresponding endocrine therapy.

AR is essential to maintain the physiological functions and development of prostate and is also involved in carcinogenesis, disease progression and management of prostate cancer, which have been intensively investigated for decades (18). Our previous study showed that CMG2 is expressed in both normal prostate and prostate cancerous tissues (8). In the current study, immunohistochemical staining showed reduced expression of CMG2 in metastatic tumours from prostate cancer in comparison with primary tumours. Similar to the ER, AR is also a classical nuclear

receptor. The finding of ER-mediated repression of CMG2 in breast cancer inspired us to investigate whether AR also mediates the regulation of CMG2 in prostate cancer thus contributing to disease progression. Compared with AR (–) prostate tumours, lower CMG2 expression was associated with lymph node metastasis and a higher Gleason score in AR (+) prostate cancers, suggesting that AR-associated down-regulation of CMG2 is involved in the progression of prostate cancer being similar to the role of ER-regulated CMG2 in breast cancer. Further analysis showed that CMG2 is also inversely correlated with AR in the prostate cancer tumours. This inverse correlation was also evident in the quantitative analysis of CMG2 transcripts in prostate cancer cell lines, which showed a lower expression of CMG2 in AR (+) prostate cancer cell lines (LNCaP and VCaP). In order to demonstrate whether the difference in CMG2 expression was due to the presence of AR, AR was forcibly over-expressed in AR (–) cell lines PC-3 and DU-145.
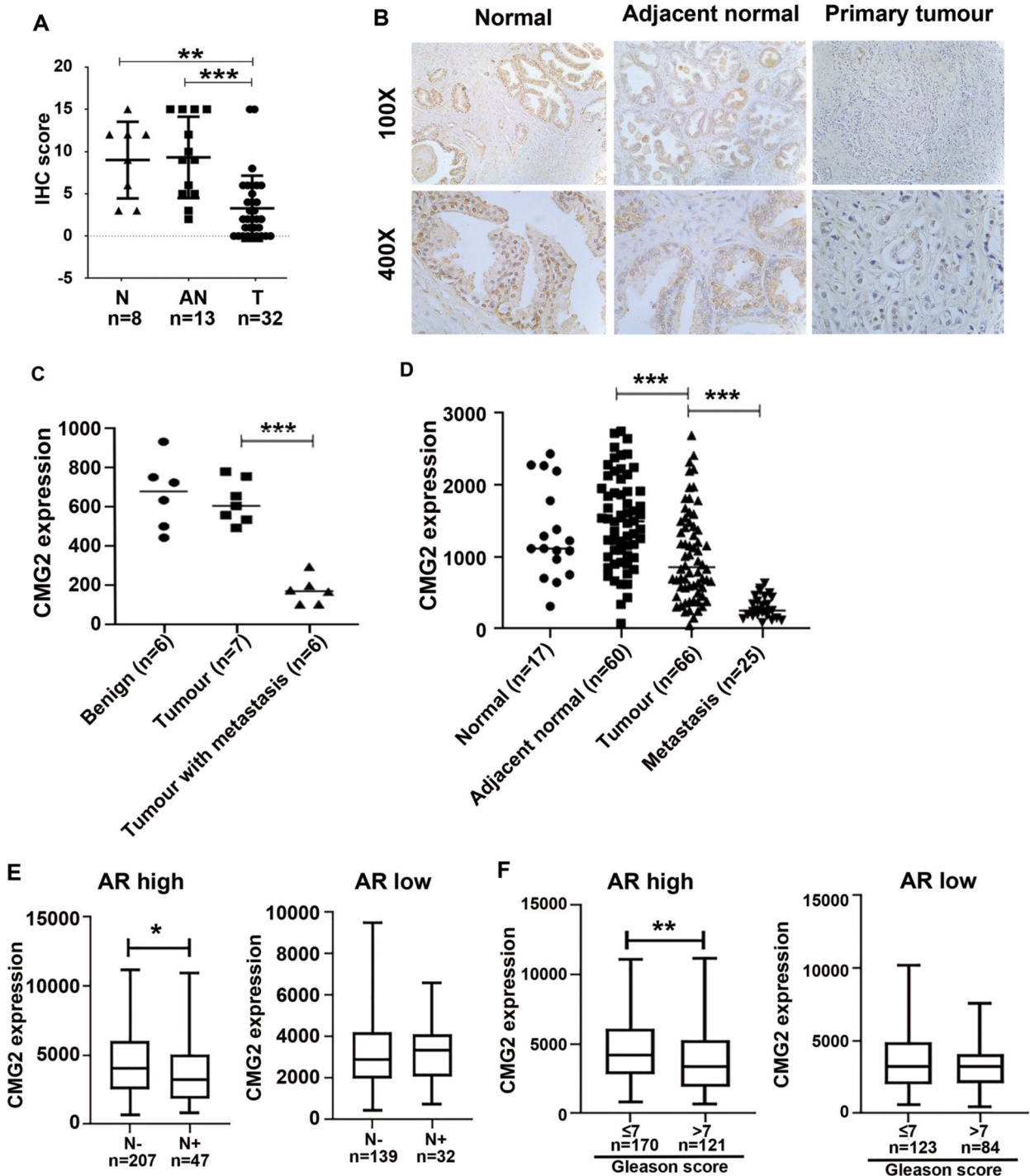
Figure 3. *Capillary morphogenesis gene 2 (CMG2) is associated with the progression of androgen receptor (AR)+ prostate cancer. (A) Immunohistochemical staining for CMG2 was performed on a tissue microarray (PR8011a, Biomax) comprising tumours (T), adjacent normal prostatic tissues (AN), and normal prostate tissues (N). (B) Representative images taken from normal, adjacent normal and tumour samples. (C) Expression of ANTXR2 was analysed in a gene expression array dataset (GSE3325, CMG2ANTXR2 gene ID:238050_at, GSE3325). Expression of ANTXR2 was observed in localised primary prostate tumours in comparison with its expression in benign prostate tissues, whilst a further reduction was seen in primary tumours which developed distant metastases. (D) Expression of ANTXR2 in metastases of prostate cancer was analysed in a gene array GEO dataset (GSE6919, CMG2ANTXR2 gene ID: 58617_at, GSE6919). (E) Implication of CMG2 in lymph node metastasis was analysed in tumours of TCGA dataset. The tumours were separated into two groups: AR-high and AR-low groups according to AR expression (cut off value=400.39). (F) Association between CMG2 and Gleason score was also analysed in tumours with different AR expression.*
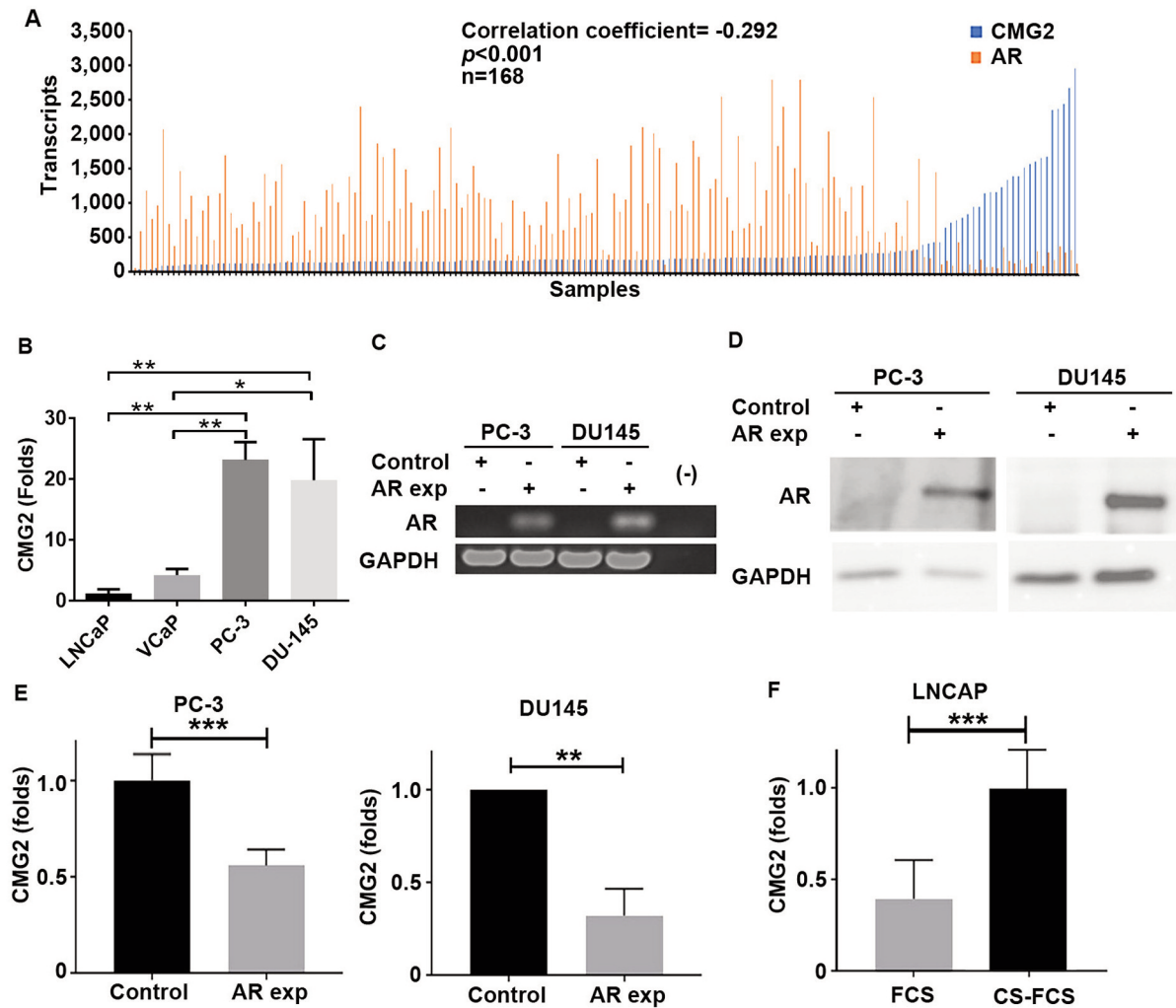
Figure 4. *Androgen receptor (AR) mediates a regulation of capillary morphogenesis gene 2 (CMG2) in prostate cancer cells. (A) Correlation between CMG2 and AR as analysed in a gene array dataset (GSE6919, CMG2:51714_at, AR: 52851_at). (B) Expression of CMG2 in AR positive prostate cancer cell lines (LNCAP and VCAP), and AR negative cell lines (PC-3 and DU-145) was determined using QPCR. Fold changes in CMG2 transcripts normalised against GAPDH. AR was over-expressed in both PC-3 and DU-145 cell lines using empty lentiviral vectors or lentiviral vectors carrying the coding sequence of human AR, which is named as AR exp. The expression of AR was determined using both conventional PCR (C) and western blot (D). (E) CMG2 expression was determined in both PC-3 and DU-145 cell lines with AR over-expression using QPCR. (F) CMG2 expression in LNCaP cells following a 24-h deprivation from steroid hormones using charcoal stripped serum was determined using QPCR. *p<0.05, **p<0.01, ***p<0.001.*

CMG2 transcripts were decreased significantly following the AR over-expression. This is further supported by the finding of increased CMG2 expression in the LNCaP cells under androgen deprivation. It suggests that AR mediated repression of CMG2 expression in prostate cancer cells, which is similar to the ER-mediated regulation of CMG2 in breast cancer.

Conclusion

To date, little is known about the mechanism CMG2 deregulation in malignant tumours. The present study demonstrated for the first time that both ER and AR mediate the down-regulation of CMG2 in breast and prostate cancer, respectively. Sex hormone receptors mediate the down-regulation of CMG2, which is involved in disease progression. Further investigation will highlight its potential in the disease management of both breast cancer and prostate cancer when endocrine therapy is applied.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

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