The prognostic significance of specific *HOX* gene expression patterns in ovarian cancer

Zoe Kelly[1]

Carla Moller-Levet[2]

Sophie McGrath[1]

Simon Butler-Manuel[4]

Andrzej M. Kierzek[2]

Hardev Pandha[1]

Richard Morgan[3]

Agnieszka Michael[1]

Thumuluru Kavitha Madhuri[4]

[1] Oncology, School of Biosciences and Medicine, FHMS, Leggett Building, Daphne Jackson Road, University of Surrey, Guildford GU2 7WG

[2] Computational & Systems Biology, School of Biosciences and Medicine, FHMS, University of Surrey, Guildford, GU2 7TE

[3] ICT building, Institute of Cancer Therapeutics, University of Bradford, West Yorkshire, BD7 1DP

[4] Royal Surrey County Hospital, Egerton Road, Guildford GU2 7XX

Correspondence: Dr Zoe Kelly

E-mail: z.l.kelly@outlook.com

Novelty and impact statement

This is the first comprehensive analysis comparing *HOX* gene expression in ovarian cancer to normal ovarian and fallopian tube tissue. *HOX* expression has been analysed along with the clinico-pathological features of each patient, identifying the significance of specific *HOX* genes relating to poor overall survival. This is the first study to analyse changes in *HOX* expression with the development of platinum resistance, finding increased expression of specific genes after resistance occurs, identifying them as potential therapeutic targets.

Abstract

HOX genes are vital for all aspects of mammalian growth and differentiation, and their dysregulated expression is related to ovarian carcinogenesis. The aim of the current study was to establish the prognostic value of *HOX* dysregulation as well as its role in platinum resistance. The potential to target HOX proteins through the HOX/PBX interaction was also explored in the context of platinum resistance. *HOX* gene expression was determined in ovarian cancer cell lines and primary EOCs by QPCR, and compared to expression in normal ovarian epithelium and fallopian tube tissue samples. Statistical analysis included one-way ANOVA and t-tests, using statistical software R and GraphPad. The analysis identified 36 of the 39 *HOX* genes as being overexpressed in high grade serous EOC compared to normal tissue. We detected a molecular *HOX* gene-signature that predicted

poor outcome. Overexpression of *HOXB4* and *HOXB9* was identified in high grade serous cell lines after platinum resistance developed. Targeting the HOX/PBX dimer with the HXR9 peptide enhanced the cytotoxicity of cisplatin in platinum-resistant ovarian cancer. In conclusion, this study has shown the *HOX* genes are highly dysregulated in ovarian cancer with high expression of *HOXA13*, *B6*, *C13*, *D1* and *D13* being predictive of poor clinical outcome. Targeting the HOX/PBX dimer in platinum–resistant cancer represents a potentially new therapeutic option that should be further developed and tested in clinical trials.

Keywords: Ovarian cancer, *HOX* genes, survival, prognosis, targeted treatment

Introduction

Ovarian cancer is the 5th leading cause of cancer death in women in the western world and it is estimated there were 22,280 new cases and 15,500 deaths due to the disease in the US in 2012 (1). It is the most lethal of the gynaecological malignancies largely due to late diagnosis. Standard treatment involves debulking surgery followed by a combination of taxane and platinum-based therapy. Initially most women respond to platinum-based therapy, but the majority suffer disease recurrence due to drug resistance. It is therefore essential to introduce new therapeutic approaches to improve treatment at diagnosis and/or provide an effective second line treatment.

There are different types of ovarian cancer classified by the cell type they originate from. The most common form, accounting for more than 90% of ovarian cancers, is epithelial ovarian cancer (EOC), and the high grade serous (HGS) subtype accounts for approximately 80% of EOC cases.

The epithelial ovarian tumours undergo Müllerian differentiation, which suggests that differentiation-regulatory factors may contribute to their progression. This mechanism has

been shown to involve homeobox (*HOX*) genes (2, 3) which play important roles in tissue differentiation during embryonic development. The *HOX* genes constitute a family of transcription factors, and in mammals 39 *HOX* genes have been identified. They are organised into 4 paralogous clusters (A, B, C and D) located on 4 different chromosomes. During development of the female reproductive system four *HOX* genes, *HOXA9, HOXA10, HOXA11,* and *HOXA13* are expressed uniformly along the Müllerian duct axis. *HOXA9* becomes expressed in the fallopian tubes, *HOXA10* is expressed in the developing uterus, *HOXA11* in the lower uterine segment and cervix and *HOXA13* in the upper vagina (4). It is thought that inappropriate expression of these genes is an early step in epithelial ovarian neoplasia as they induce aberrant epithelial differentiation. Studies which have analysed *HOX* gene expression in ovarian cancer cell lines and a small number of tumours have found dysregulated expression of certain *HOX* genes compared to normal tissue (5). Numerous studies have also demonstrated dysregulated *HOX* gene expression in other cancers such as lung, prostate, breast, colon and bladder cancer (6-9). The recent genomic analysis of HGS ovarian cancer (HGS-OvCa) by the Cancer Genome Atlas (TCGA) researchers found a number of somatic copy number alterations with three members of the *HOXB* family, *HOXB2*, *B5* and *B8* among the focally amplified regions. The group divided HGS ovarian cancer into four expression subtypes ‘immunoreactive’, ‘differentiated’, ‘proliferative’ and ‘mesenchymal’ on the basis of gene expression, and high expression of *HOX* genes was a characteristic of the mesenchymal subtype (10). High expression of *HOX* genes makes them a potential target for therapeutic intervention. One possible method is the use of a peptide that disrupts the interaction between HOX proteins and co-factor PBX. HXR9 is a small peptide designed to mimic the hexapeptide sequence found in HOX proteins of paralogue groups 1- 9 (11), therefore acting as a specific competitive inhibitor of the HOX/PBX interaction preventing the subsequent binding of the HOX/PBX dimer to target DNA sequences. This in effect inhibits the transcription of target genes. Previous studies have shown that HXR9 is capable of blocking this interaction *in vitro* and *in vivo* (11-13) and antagonising the HOX/PBX interaction induces apoptosis (11-15).

The role of aberrant *HOX* dysregulation in EOC is not yet understood. The aim of the current study was to establish the prognostic value of *HOX* dysregulation as well as its role in developing platinum resistance. The potential to target *HOX* function through the HOX/PBX interaction was also explored in the context of platinum resistance (13).

Material and methods

Cell lines and reagents

The human ovarian adenocarcinoma-derived HGS cell line SKOV-3, clear cell carcinoma derived cell line TOV-21G and the endometrioid carcinoma derived cell line TOV-112D were obtained from the American Type Culture Collection (LGC Promochem, Teddington, UK). The SKOV-3 cell line has since been reclassified as an endometrioid subtype due to the lack of a p-53 mutation and the presence of the endometrioid associated ARID1A mutation (16). Therefore, the SKOV-3 cell line will be considered as an endometrioid cell line in this paper. SKOV-3 cells were cultured in McCoys's 5A modified medium (Sigma, Poole, UK) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Invitrogen Ltd, Paisley, UK). TOV-112D and TOV-21G cells were cultured in 1:1 mixture of MCDB 105 medium (Sigma) supplemented with 1.5 g/L sodium bicarbonate and Medium 199 (Invitrogen), with 15% heat-inactivated FBS (Invitrogen). The epithelial HGS carcinoma cell line derived from peritoneal ascites COV-318 and the paired HGS ovarian carcinoma cell lines PEO1, PEO4, PEO14 and PEO23 were obtained from the HPA Cell Culture Collection (HPA, Salisbury, UK) (17). These cell lines were authenticated by either STR profiling (DDC Medical, OH, USA) or LCG Standards (Middlesex, UK). COV-318 cells were cultured in DMEM medium (Sigma) with 10% heat-inactivated FBS and 2mM glutamine (Sigma). PEO cell lines were maintained in RPMI1640 media with 10% heat-inactivated FBS. All media was supplemented with 1% penicillin (10,000 U/ml) / streptomycin (10 µg/ml) (Sigma). Cell

cultures were maintained at 37^{o}C in a humidified, 5% CO_2 incubator. Cisplatin sensitivity of cell lines was verified by MTS assay after 72 hour cisplatin treatment.

RNA isolation, cDNA production and quantitative Real Time PCR (qRT-PCR)

Two total RNA samples from normal human ovarian tissue were purchased from OriGene (Cambridge, UK). All cell lines were grown in normal growth medium in 6-well plates at a density range to ensure overnight growth resulted in until 80% confluency before RNA extraction took place. RNA was isolated from cell lines using the RNeasy® Plus Mini Kit (Qiagen Ltd, Crawley, UK) following the manufacturer's instructions. This included the use of gDNA eliminator columns to remove genomic DNA contamination. Total RNA extracted from 20–30mg ovarian tumour or ovarian normal tissue stored in RNA*later*® (Sigma) was isolated using the gentleMACS dissociator followed by RNA extraction using the RNeasy® Plus Mini Kit (Qiagen). RNA purity was verified by the 260nm/280nm absorbance ratio, measured using the Nanodrop (Thermo Fisher, MA). Ratios of 1.9-2.0 were considered 'pure' RNA as described by manufacture. cDNA was synthesised from RNA using the Cloned AMV First Strand Synthesis Kit (Invitrogen) following the manufacturer's protocol. qRT-PCR was performed using the Stratagene MX3005P Real Time PCR machine (Agilent Technologies UK Ltd, Stockport, UK) and SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma). Oligonucleotide primers were designed to facilitate the unique amplification of β-actin and each *HOX* gene. Melt curves and gels were run originally to validate the primers and check for single bands of the correct product size. Relative expression was calculated using the Livak comparative Ct method (18).

Synthesis of HXR9 and CXR9 peptides

HXR9 is an 18 amino acid peptide consisting of the previously identified hexapeptide sequence that can bind to PBX and nine C-terminal arginine residues (R9) that facilitate cell entry (11). The N-terminal and C-terminal amino bonds are in the $_{D}$-isomer conformation, which has previously been shown to extend the half-life of the peptide to 12 h in human serum (11). CXR9 is a control peptide that lacks a

functional hexapeptide sequence but still includes the R9 sequence. All peptides were synthesized using conventional column based chemistry and purified to at least 80 % (Biosynthesis Inc., USA).

Analysis of cell death and apoptosis

Cells were plated in flat bottomed 96-well plates and incubated for 24 hours until 70% confluent. Cells were treated with HXR9 or CXR9 at a range of dilutions for 2 hours. Cell viability was measured via the MTS assay (Promega, Southampton, UK) according to the manufacturer's instructions. To detect morphological changes consistent with apoptosis, cells were plated in 24-well plates and incubated overnight to reach 70% confluency. Cells were then treated for 2 hours with 2% FBS media, the control peptide CXR9 or the active peptide HXR9 at the IC_{50} (Concentration of drug needed to induce 50% cell death, as determined by the MTS assay) and double the IC_{50}. Cells were then harvested by incubating in trypsin-EDTA (Sigma) at 37^{o}C until detached and dissociated. Apoptotic cells were identified using a Beckman Coulter Epics XL flow cytometer (argon laser, excitation wavelength 488 nm, FL-2 and FL-4 detectors) and the Annexin V-PE apoptosis detection kit (BD Pharmingen) as described by the manufacturer's protocol. Caspase-3 activity was measured using the EnzCheck Caspase-3 Assay Kit (Molecular Probes), using the protocol defined by the manufacturer.

Calculating synergy

To measure synergistic interaction between HXR9 and cisplatin, cells were plated in a 96-well plate and treated with either HXR9 or cisplatin alone or in combination at concentrations of the drugs IC_{50} and ± 2-, 4- and 8- fold this concentration. Cell viability was then measured by the MTS assay (as described above) and the presence of synergy was analysed based on the Chou-Talalay method using CalcuSyn version 2.0 software (Biosoft, Stapleford, UK) (19). The interaction between HXR9 and cisplatin was quantified by determining the combination index (CI). Using this method, CI < 1 indicates synergism, CI = 1 indicates an additive effect antagonism (CI > 1) between drugs.

Clinical data

A cohort of 99 patients with corresponding age, stage, time to progression (TTP), overall survival (OS), histology and chemotherapy information was used in the analysis of primary ovarian tumours (Supplementary Table 1). Fresh biopsy tissue specimens were obtained during surgery from human subjects with ovarian cancer or other gynaecological conditions from the Royal Surrey County Hospital, Guildford following informed consent and ethical approval. Samples were immediately stored in RNA*later*® and stored at -20^{o}C for later use. Each biopsy was confirmed by a pathologist to be either cancerous of ovarian origin or normal ovarian tissue. OS and TTP were measured from the date of diagnosis. The duration of OS was measured up to the date of death or, for patients still alive the 1st October 2012, when statistical analysis was performed. The duration of TTP was the minimum amount of time until clinical progression, or death. Only cases where causes of death were due to disease were used to calculate OS. *HOX* gene expression was obtained by qRT-PCR and values were normalised to housekeeping gene β-actin. All sample and data collection received an ethical approval by the institutional ethics committee (MREC-09/H1103/50).

Mouse i*n vivo* study

All experiments were conducted in accordance with the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the Welfare of Animals in Experimental Neoplasia (20) and were approved by the University of Surrey Ethics Committee. The mice were kept in positive pressure isolators in 12 hour light/dark cycles and food and water were available ad libitum. Six-8 week old female balb/C NUDE mice (Charles River, Kent, UK) were inoculated subcutaneously with a suspension 100µL Hanks media (Sigma) containing 10^6 SKOV-3 cells in 50% matrigel (BD Bioscience). Once tumours reached a volume of approximately 100mm^3, mice were randomised into 4 treatment groups, each containing 10 mice: PBS alone, Cisplatin alone, HXR9 alone, Cisplatin and HXR9 in combination. Mice in the HXR9 group received an initial dose of

100mg/kg HXR9 intratumorally (IT), with subsequent dosing of 10 mg/kg twice weekly. The cisplatin treatment group received a weekly dose of 3mg/kg via intraperitoneal injection (IP). PBS was used as a control. Drug concentrations were used based on previous experiments (13). The mice were monitored carefully for signs of distress, including behavioural changes and weight loss.

Statistical analysis

All data analysis and manipulation of primary ovarian tumours were performed using R (an integrated set of software tools for data manipulation, calculation and graphical display).

Four test statistics were used to evaluate the change of gene expression. For variables with two groups (i.e. Age, OS and chemotherapy) the t-test was used for parametric analysis and the Mann-Whitney test was used as a non-parametric analysis. For variables with three or more groups (i.e. TTP and Stage) the one-way ANOVA was used for parametric analysis and the Kruskal-Wallis was used as a non-parametric analysis. Differential expression and interactions based on ANOVA. The Benjamini and Hochberg and the Bonferroni correction was applied to cell line data and ovarian tumour data, respectively, to account for multiple testing. Principle component analysis (PCA) was performed and the first two principle components are plotted. The heatmaps include row Z-score transformation (genes), and are plotted in red–blue colour scale with red indicating high expression and blue indicating low expression. Analysis of OS was calculated using the Kaplan-Meier method using GraphPad PRISM Version 5.0 (GraphPad Software). Hazard ratio (HR) and confidence intervals (CI) were calculated using the Log rank model.

Results

HOX gene expression in ovarian cancer cell lines and normal ovarian and fallopian tube tissue

In order to evaluate the changes in *HOX* gene expression in EOC we compared the relative expression of all 39 *HOX* genes in normal ovarian and fallopian tube tissue to a number of ovarian cancer cell lines.

The *HOX* expression profile was analysed in a panel of 5 HGS ovarian cancer cell lines, 2 endometrioid cell lines and 1 clear cell carcinoma cell line and compared with 10 normal ovarian and 3 fallopian tube tissue samples. A highly dysregulated pattern of *HOX* gene expression was found in the EOC cell lines whereas normal tissue showed very little or no *HOX* gene expression (Figure 1).

The HGS cell lines showed marked dysregulation but this varied significantly across the panel. The COV-318 (HGS) cell line showed two *HOX* genes, with *HOXA9*, being significantly upregulated, whilst the PEO14 (HGS) cell line had 23 *HOX* genes that were significantly upregulated when compared to normal tissue.

HOX expression in platinum sensitive and resistant ovarian cancer cell lines

To evaluate differences in *HOX* expression between platinum sensitive and resistant EOC, 2 paired HGS cancer cell lines derived from patients with platinum sensitive and resistant disease were analysed. Each pair was acquired from separate patients at the time when the tumour was deemed clinically sensitive to platinum and at a later time-point after developing platinum resistance. PEO1 and PEO14 - platinum sensitive cell lines, were compared with PEO4 and PEO23, platinum resistant cell lines, respectively (21). We found significant differences in the *HOX* expression profile in platinum resistant and platinum sensitive cell lines. The PEO4 (platinum resistant cell line) showed a significant increase of *HOXB3* and *HOXB4* gene expression compared to its paired sensitive cell line, PEO1. PEO23 (platinum-

resistant) also has a relatively higher expression of *HOXB4* when compared to its platinum-sensitive couterpart-PEO14, and in addition, elevated expression of *HOXB9*. Cell line gene expression data was pooled according to platinum sensitivity status and the resistant cell lines showed an overall higher *HOX* expression compared to normal and sensitive cell lines (Figure 2).

HOX expression in primary EOC

To comprehensively evaluate *HOX* gene expression profiles in clinically relevant HGS EOC we analysed tumours from a cohort of 73 HGS ovarian cancer patients and compared it to 10 normal ovarian and 3 fallopian tube tissue samples (patients' characteristics are summarized in Table 1). HGS ovarian tumours exhibited a significant upregulation in the expression of 36 of the 39 *HOX* genes when compared to their expression in normal tissue samples (Figure 3). The strongly overexpressed genes included *HOXA9* ($p = 1.86 \times 10^{-8}$), previously reported to be related to the HGS histotype (3), however, *HOXA3* was expressed to a far higher level, ($p = 9.55 \times 10^{-10}$).

There were significant differences in *HOX* expression profiles between the HGS and endometrioid histological subtypes with up-regulation of *HOXA7, A9*, *A10*, *A13*, *B1*, *B4*, *B5*, *B13*, *C9*, *C13, D9* and *D10* in the endometrioid samples. *HOXB2* was the only gene to show a significant difference between HGS and clear cell carcinomas, although this might reflect the small sample size.

A 5-_HOX_ gene signature predicts poor OS

The *HOX* expression profile in HGS EOC was subsequently correlated with clinical characteristics such as age, stage, TTP and OS.

The *HOX* expression profile in EOC also correlated with OS. We found that 5 *HOX* genes: *HOXA13*, *B6*, *C13*, *D1* and *D13* were expressed significantly more strongly in the tumours of patients with poor survival with higher expression of these genes found in all deceased

patients. Each of these genes were individually analysed by the Kaplan-Meier method and the result from the analysis are summarised in Table 1.

Targeting the HOX/PBX dimer in platinum-resistant EOC

The aberrant *HOX* expression found in EOC makes them a potential therapeutic target. As the function of *HOX* genes is partly based on the binding of HOX proteins to the PBX and MEIS co-factors, targeting these co-factors could impair the oncogenic potential of HOX. PBX and MEIS proteins are present in both the nucleus and cytoplasm in ovarian carcinomas, however only MEIS is expressed in normal ovarian epithelia (22). These co-factors are important for ovarian carcinogenesis, most likely through potentiating the function of HOX proteins. A peptide called HXR9 has been designed to target the interaction between HOX proteins (members of paralogue groups 1-9) and PBX (11). This drug has been shown previously to induce apoptosis in cancer cells with highly dysregulated *HOX* expression profiles (11, 12, 14, 15), including the ovarian cancer cell line SKOV-3 (13). SKOV-3 is platinum-resistant, although its origin has recently been questioned (16).

In view of the gross *HOX* dysregulation pattern seen in platinum-resistant tumours we have used HXR9 alone and in combination with cisplatin to evaluate its efficacy in this setting. HXR9 and its control peptide –CXR9 (which has an identical polyarginine cell penetrating sequence to HXR9 but has a single alanine substitution in its hexapeptide sequence that renders inactive) have been described previously (11). All cell lines treated with HXR9 demonstrated an increase in *cFOS* expression, which is thought to be at least partly responsible for HXR9-induced cell death (data not shown) (11). When analysed with flow cytometry for Annexin-V-PE there was a significant increase in the number of cells in late apoptosis after HXR9 treatment compared to untreated cells (Figure 4a, b). Previous publications have also demonstrated the apoptosis inducing capacity of HXR9 in ovarian cancer cell lines showing PARP cleavage and caspase-3 activity in treated cancer cells (11). The *in vitro* experiments showed that HGS cell lines were all sensitive to HXR9 treatment but

not to CXR9 and when combined with cisplatin there was synergy between HXR9 and cisplatin as shown in Supplementary Table 2. There was also enhanced cell killing *in vivo* using a combination of HXR9 and cisplatin over each drug used alone when treating mice bearing SKOV-3 tumours (Figure 5). Despite a good synergy effect seen *in vitro*, the effect *in vivo* was not as powerful and the combination of HXR9 and cisplatin was only marginally more active than HXR9 alone. This however may be cell line dependent. Combined HXR9 and cisplatin provided a survival advantage, with a hazard ratio of 1.98 (95% CI, -0.88–6.58; $p = 0.098$) determined by the Log-rank model.

Discussion

This study confirms that *HOX* genes are highly dysregulated in ovarian cancer and that targeting the HOX/PBX interaction in platinum resistant tumours is of therapeutic value. Little to no *HOX* expression was found in normal ovarian tissue, whereas increased expression of certain groups of *HOX* genes was found in the majority of ovarian cancers.

The HGS carcinoma subtype shows the highest degree of heterogeneity in *HOX* expression for both cell lines and primary tumours, whereas endometrioid subtypes show a very distinct *HOX* expression profile. The HGS histological subtype is known to have a very heterogeneous nature, exhibiting a wide range of underlying genetic alterations, which may explain this variation. However, the functional redundancy between *HOX* genes may mean the net effect of *HOX* overexpression is similar even in cells expressing different sets of *HOX* genes (23).

Previous studies have shown that the over-expression of specific *HOX* genes determines the histological subtype, with *HOXA9* being overexpressed in HGS subtypes, *HOXA10* in endometrioid and *HOXA11* in mucinous (3). In this study we found that *HOXA9* is overexpressed in only 3 of the 8 HGS cell lines, but is also expressed in the clear cell and endometrioid cell lines. With regards to the primary tumours, *HOXA9* is significantly overexpressed in the HGS samples; however *HOXA10* and *HOXA11* are also expressed at

high levels in HGS tumours, which has not been previously reported. The endometrioid cell lines show an overall higher level of *HOX* expression than the HGS cell lines, including *HOXA9* and *HOXA10*.

HOXA7 has been previously reported to play a role in the differentiation of ovarian surface epithelia (OSE) into EOC (24). We found that *HOXA7* is overexpressed in the HGS cancers as well as in the endometrioid carcinomas compared to normal ovarian tissue. In addition, *HOXA13* is overexpressed in the endometrioid tumours. This suggests that the *HOXA* genes play a role in the determination of histological subtypes, but the differences in expression are not as clear as previously suggested by Cheng *et al*. The high expression of *HOXA10* in the endometrioid cell lines and primary tumours does support a role for this gene in the differentiation of endometrioid tumours; however the high level of heterogeneity in cancer calls for caution in the interpretation of the results as the level of gene expression may differ in individual tumour samples.

Although the function of the *HOX* genes in cancer remains unclear, there have been reports that they act as tumour suppressor genes or oncogenes. In ovarian cancer both *HOXB7* and *B13* expression has been linked to the invasive characteristics of ovarian cancer cells (25), and *HOXB7* has been shown to be a regulator of bFGF- a potent mitogenic and angiogenic factor (26) and involved in double strand break repair (27), whereas *HOXB13* promotes cell proliferation (28).

We found that 9 out of the 10 *HOXB* genes were upregulated, the most significant being *HOXB4*, *B5*, *B7* and *B13*. *HOXB4* upregulation has been shown to be associated with the development of platinum resistance in cell lines, and its over-expression in ovarian cancer has been reported previously in a relatively small study using only 4 cell lines and 7 ovarian cancer tumour samples (29), but no oncogenic function for this gene has been proven. *HOXB4* has been implicated as a cancer-related gene in other malignancies, including breast cancer, leukaemia and lung cancer (30-32). The recent genomic analysis of HGS

ovarian cancer by the Cancer Genome Atlas (TCGA) researchers found a number of somatic copy number alterations and three members of the *HOXB* family, *HOXB2*, *B5* and *B8* were among the focally amplified regions (33) further supporting a possible oncogenic role of *HOXB* genes in ovarian cancer and emphasising the overlapping functions which exist between *HOX* genes (34).

Significant differences in *HOX* gene expression were found between platinum sensitive and resistant cell lines. Platinum-resistant cell lines show upregulation of *HOX* genes from the *HOXB* cluster. Although there was a difference between the three paired cell lines tested, *HOXB4* and *HOXB9* overexpression was common in two of the three cell lines (when compared to the platinum-sensitive counterpart). These results therefore demonstrate that *HOXB* genes are likely to play a role in developing platinum resistance; although further work is needed to understand the mechanism of this interaction.

Survival analysis revealed a cluster of 5 *HOX* genes, *HOXA13*, *B6*, *C13*, *D1* and *D13,* that was strongly associated with a poor OS in HGS patients. *HOXA13* is usually expressed in the upper vagina (4) playing a role in Müllerian duct differentiation during development, but has been reported to be overexpressed in ovarian cancer cell lines (25). *HOXA13* was linked to poor OS in oesophageal squamous cell carcinoma patients, and the same study found its expression in cell lines enhanced tumour growth *in vitro* and *in vivo* (35). High-throughput microarray analysis of gastric cancer patients revealed *HOXA10* and *A13* over-expression with *HOXA13* upregulation significantly associated with an aggressive phenotype, and a prognostic marker for poor OS (36). Highly deregulated expression of the *HOXA* cluster has also been found in hepatocellular carcinoma (HCC), in particular *HOXA13* (37).

Up-regulation of *HOXB6* has also been reported in ovarian cancer before, in addition to *HOXB7* (38). Data from this study and previous reports of high *HOXB* expression in ovarian cancer suggests that the *HOXB* gene products play a role in ovarian tumourgenesis.

HOXC13 has a role in DNA replication (39), supporting an oncogenic function. A role in human cancer has also been reported with overexpression found in metastatic melanoma (40) and fusion with NUP98 has been associated with acute myeloid leukaemia (AML) (41). The *HOXD1* gene appears to be involved in cell differentiation (42), whereas *HOXD13* is deregulated in breast and cervical cancer and melanoma (43-45). A large *HOXD13* expression analysis by Cantile and colleagues in 79 different tumour types also supports its role in neoplastic transformation (46).

Determination of *HOX* gene dysregulation may be undertaken routinely in the clinical setting using fresh or archived patient tissue and such information could be used for stratifying patients in terms of prognosis. Furthermore, we have shown that our novel agent HXR9, a peptide capable of disrupting *HOX* gene function by inhibiting HOX binding to its co-factor, PBX, has significant anti-tumour efficacy (11-15), which is increased when used in combination with cisplatin. This synergy could be explained due to the role of *HOX* genes in DNA repair pathways (27) but further work investigating this is needed. *HOX* gene dysregulation therefore represents a potential ovarian cancer target with a low likelihood of cross resistance to conventional chemotherapeutic agents. Both HXR9 and small molecule inhibitors of the HOX/PBX dimer are currently being evaluated as novel cancer agents in preclinical models.

Conclusion

This comprehensive analysis of *HOX* gene expression in ovarian cancer cell lines and primary ovarian tumours demonstrates that these genes are profoundly dysregulated compared to normal ovary. Increased expression of *HOXA13*, *B6*, *C13*, *D1* and *D13* in EOC patients is associated with a poor prognosis and a more aggressive malignancy. It is possible to target HOX function by disrupting its binding to PBX, and further development of therapeutic compounds to achieve this is warranted.

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Conflicts of interest statement

The authors report no conflicts of interest.

Figure legends

Figure 1

***HOX* expression in ovarian cancer cell lines of high grade serous (HGS), endometrioid and clear cell carcinoma subtypes and normal ovarian and fallopian tube tissue.** Heat map showing differential *HOX* expression between 5 HGS, 2 endometrioid and 1 clear cell ovarian carcinoma cell lines and 10 normal ovarian and 3 fallopian tube tissue samples. Expression of each gene was determined by quantitative PCR (qRT-PCR) from at least three independent experiments and expression is relative to the house keeping gene β-actin.

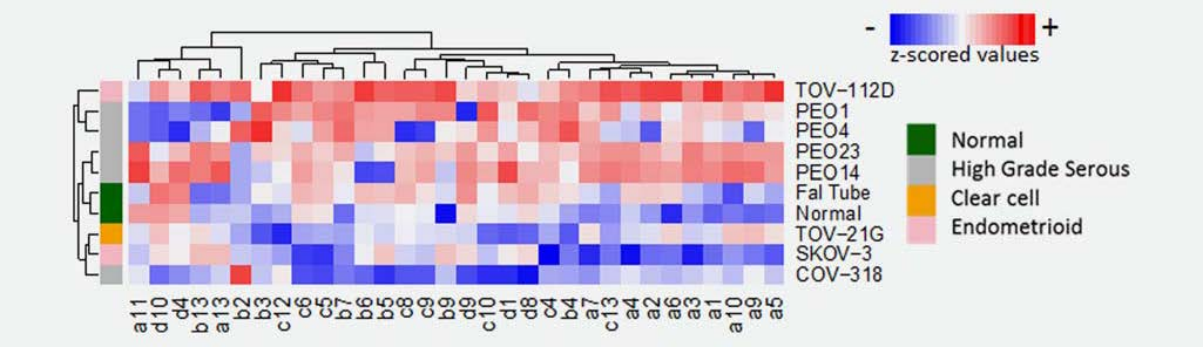


Figure 2

***HOX* gene expression of paired platinum sensitive and platinum resistant ovarian cancer cell lines.** The comparison of *HOX* gene expression profiles between patient derived cell lines before (PEO1 and PEO14) and after (PEO4 and PEO23) developing platinum resistance. Heat map shows differential *HOX* expression between platinum sensitivity statuses of cell lines.

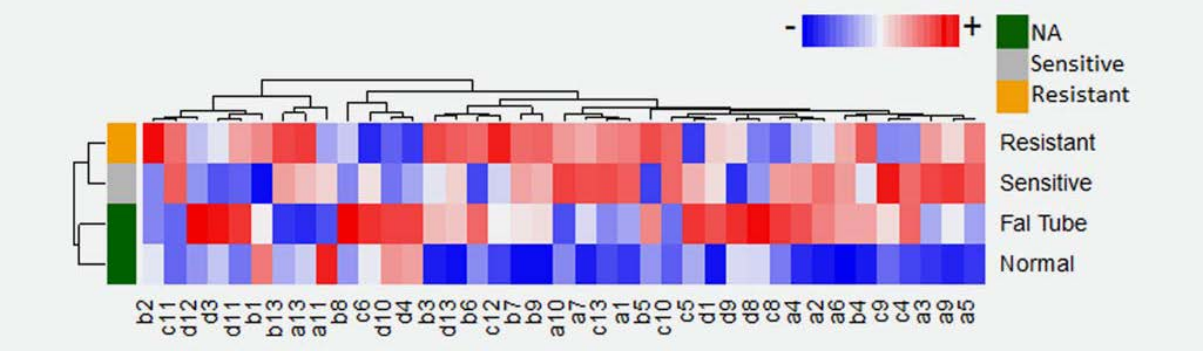


Figure 3

***HOX* gene expression in high grade serous ovarian tumours.** a) Heat map showing differentially expressed *HOX* genes between high grade serous (HGS) ovarian tumours and normal ovarian and fallopian tube tissue. *HOX* gene expression data for HGS tumours (yellow) were compared to 10 normal ovarian tissues (green) and 3 fallopian tube sample (purple) to find upregulation of 36 genes in the HGS tumours. *HOX* expression profiles were determined by quantitative PCR (qRT-PCR) and normalised to housekeeping gene β-actin. Each column represents a gene and each row represents a sample. Column-wise z-score transformation (genes) was used. Red colour for a gene indicates expression above the median and blue indicates expression below the median.

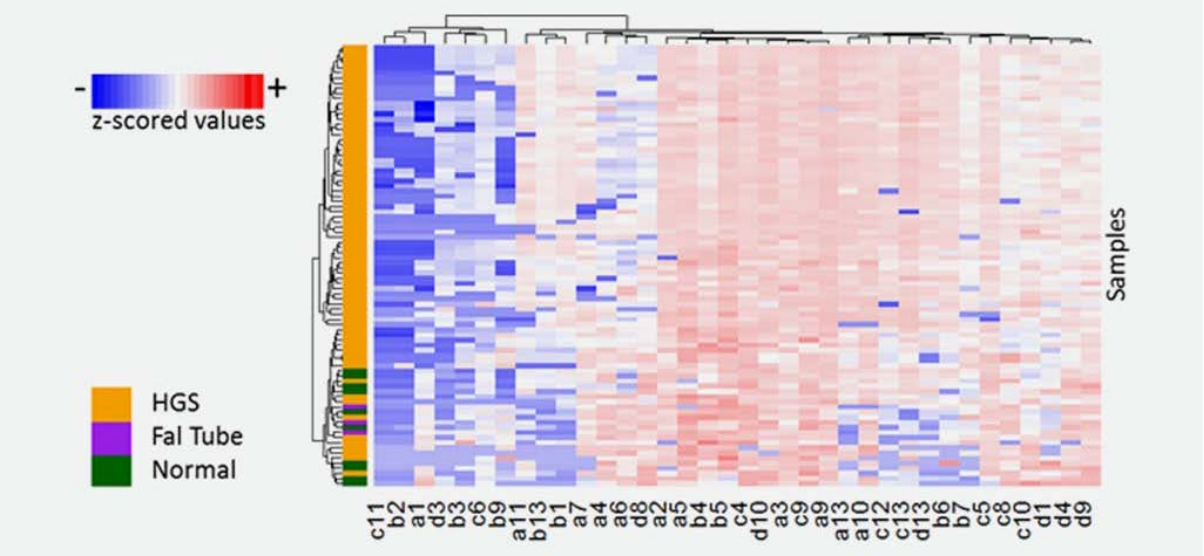
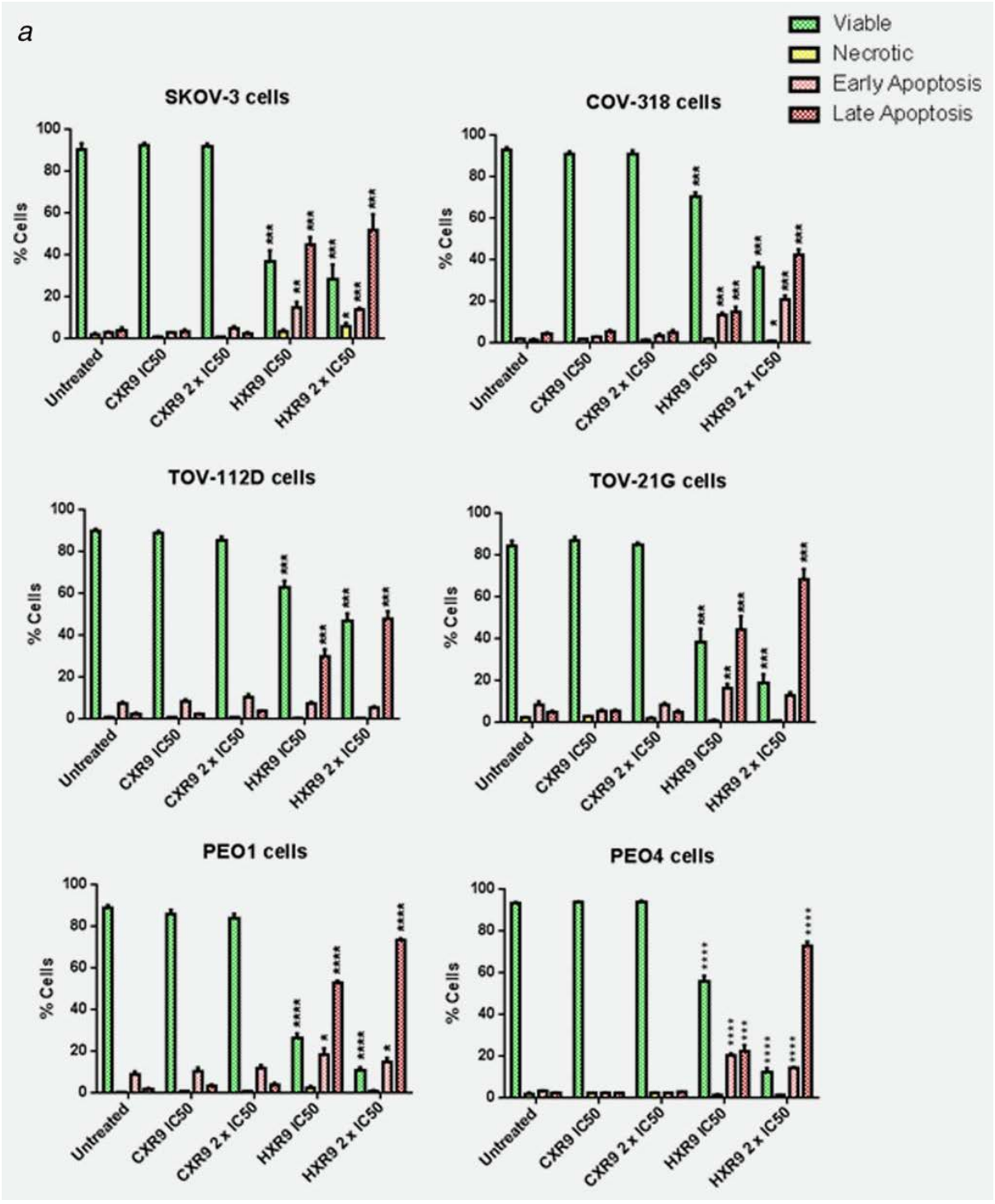


Figure 4

HXR9 induces apoptosis in ovarian cancer cell lines. Ovarian cancer cell were assessed for apoptosis or necrosis through annexin/propidium iodine staining after HXR9 treatment. a) The bargraphs show the percentage of cells in early apoptosis, late apoptosis, and necrosis, as well as viable cells, when untreated, treated at the HXR9 IC_{50} dose or double the IC_{50} dose for each cell line or equivalent CXR9 dose. Error bars show the SEM. *P*-values <0.05 are denoted as *, <0.005 ** and < 0.001 as *** with respect to untreated cells. Example flow cytometry plots for untreated; CXR9 25µM; CXR9 50µM; HXR9 25µM and HXR9 50µM treated SKOV-3 cells


a
Viable
Necrotic
Early Apoptosis
Late Apoptosis
SKOV-3 cells
COV-318 cells
TOV-112D cells
TOV-21G cells
PEO1 cells
PEO4 cells
% Cells
Untreated
CXR9 IC50
CXR9 2 x IC50
HXR9 IC50
HXR9 2 x IC50

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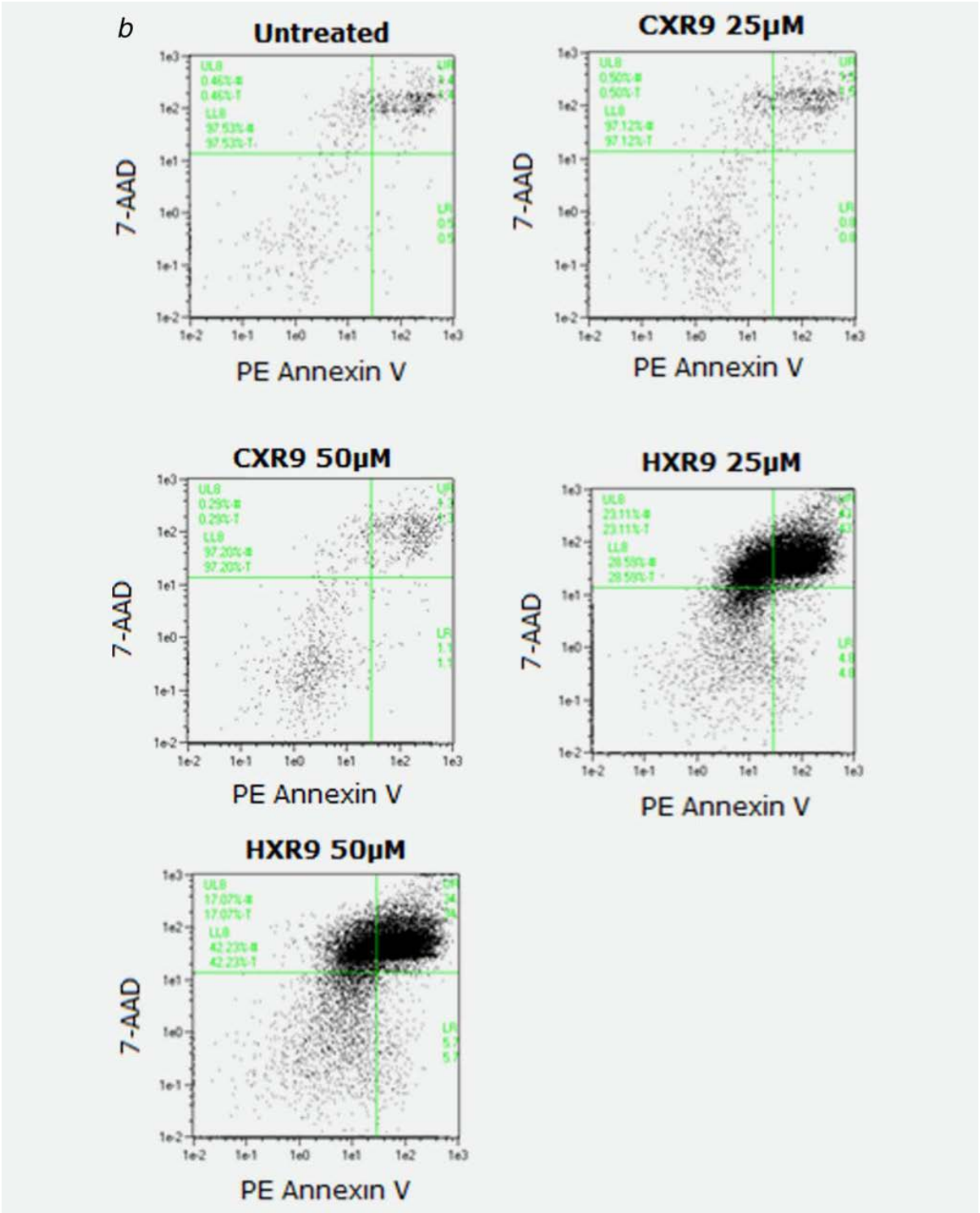

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Figure 5

***In vivo* combination study of HXR9 and cisplatin.** Antitumor activity of HXR9 and cisplatin alone or in combination against ovarian cancer (SKOV-3) xenografts. Nude female mice were inoculated SC with 1 x 10^6 SKOV-3 cells (Day 0). Treatment was initiated when tumours reached an approximate volume of 100m^3. An initial dose of HXR9 was given IT at 100 mg/kg, followed by twice weekly doses at 10 mg/kg. Cisplatin was administered IP at 3 mg/kg weekly. Combinational studies consisted of both treatments; PBS was used as a control. Arrows indicates drug administration. A minimum of 6 mice in each group was set as the cut-off point for each curve.

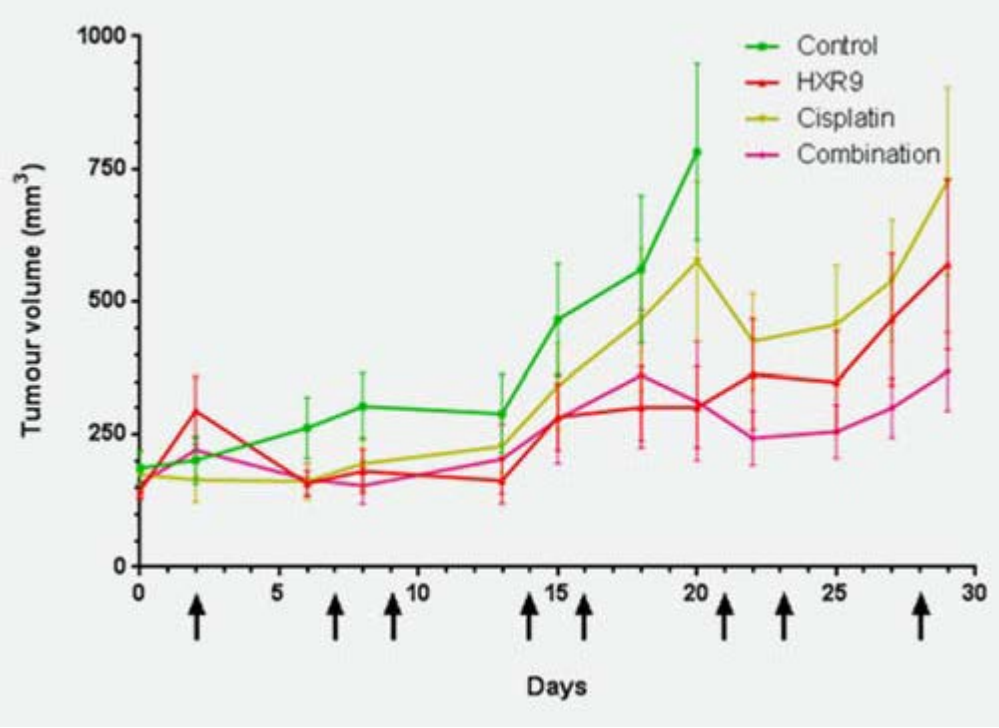

Table 1

Kaplan-Meier analysis of the 5-*HOX* gene prognostic signature showing the median overall survival for patients who do not express the gene as compared to patients whose tumours show expression of the genes listed below. Hazard ratios and Confidence intervals were calculated using the Log rank model

Gene	Median overall survival (months)	No. of patients	p values	Hazard ratio	95% Confidence interval
HOXC13	36	37	0.0128	8.264	1.396–12.75
HOXB6	36	36	0.0145	8.286	1.365–14.67
HOXA13	44	39	0.0317	4.508	1.145–12.17
HOXD13	36	37	0.0308	6.834	1.153–12.79
HOXD1	36	37	0.025	4.692	1.206–11.61

Supplementary Table 1

Patient characteristics. Tumour samples and patient information was obtained from the Royal Surrey Hospital, Guildford.

Supplementary Table 2

Synergy between HXR9 and cisplatin in ovarian cancer cell lines. Cells plated in 96-well plates were exposed to graded concentrations of HXR9 or cisplatin either alone or in combination. Cells were treated with cisplatin for a total of 72 hours and with HXR9 for 2 hours. Cell viability was then measured by the MTS assay and the presence of synergy was analysed based on the Chou-Talalay method using CalcuSyn version 2.0 software (Biosoft, Stapleford, UK) (19). The interaction between HXR9 and cisplatin was quantified by determining the combination index (CI) at ED50, ED75 and ED90 (doses which produce the cell kill in 50, 75 and 90 per cent of a population, respectively), where CI < 1 indicates synergism, CI = 1 indicates an additive effect antagonism (CI > 1) between drugs.

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