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

Zoe Kelly1, Carla Moller-Levet2, Sophie McGrath1, Simon Butler-Manuel1, Thumuluru Kavitha Madhuri3, Andrzej M. Kierzek2, Hardev Pandha1, Richard Morgan4 and Agnieszka Michael1

1Oncology, School of Biosciences and Medicine, FHMS, Leggett Building, Daphne Jackson Road, University of Surrey, Guildford, GU2 7WG
2Computational & Systems Biology, School of Biosciences and Medicine, FHMS, University of Surrey, Guildford, GU2 7TE
3Royal Surrey County Hospital, Egerton Road, Guildford, GU2 7XK
4ICT Building, Institute of Cancer Therapeutics, University of Bradford, West Yorkshire, BD7 1DP

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.

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.

Key words: ovarian cancer, HOX genes, survival, prognosis, targeted treatment

Additional Supporting Information may be found in the online version of this article.

DOI: 10.1002/ijc.30204
History: Received 5 Apr 2016; Accepted 25 Apr 2016; Online 25 May 2016
Correspondence to: Zoe Kelly, University of Surrey, Faculty of Health and Medical Sciences, Graduate School Oncology, Guildford, Surrey, United Kingdom, Tel.: [07910486222], E-mail: z.l.kelly@outlook.com

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
breast, colon and bladder cancer.\textsuperscript{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.\textsuperscript{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,\textsuperscript{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 \textit{in vitro} and \textit{in vivo}\textsuperscript{11–13} and antagonising the HOX/PBX interaction induces apoptosis.\textsuperscript{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.\textsuperscript{13}

**What's new?**

**Homebox (HOX) genes, which serve key functions in DNA repair and cell differentiation, are aberrantly expressed in ovarian cancer.** How they influence the disease, however, remains enigmatic. Here, a five-gene signature, involving elevated expression of HOXA13, B6, C13, D1 and D13, was found to predict poor clinical outcome in epithelial ovarian cancer. Meanwhile, platinum resistance in high grade serous ovarian cancer cells was linked to HOXB4 and HOXB9 overexpression. In mice, treatment with HXR9, a peptide that disrupts interactions between HOX and co-factor PBX, effectively recovered cisplatin sensitivity in resistant tumors, opening the path to novel therapeutic options in ovarian cancer.

**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 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.\textsuperscript{16} Therefore, the SKOV-3 cell line will be considered as an endometrioid cell line in this paper. SKOV-3 cells were cultured in McCoy’s 5A modified medium (Sigma, Poole, UK) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Invitrogen, 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).\textsuperscript{17} These cell lines were authenticated by either STR profiling (DDC Medical, OH) or LCG Standards (Middlesex, UK). COV-318 cells were cultured in DMEM medium (Sigma) with 10% heat-inactivated FBS and 2 mM 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°C in a humidiﬁed, 5% CO\textsubscript{2} incubator. Cisplatin sensitivity of cell lines was verified by MTS assay after 72 hr 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\textsuperscript{®} 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 20–30 mg ovarian tumour or ovarian normal tissue stored in RNAlater\textsuperscript{®} (Sigma) was isolated using the gentleMACS dissociator followed by RNA extraction using the RNeasy\textsuperscript{®} Plus Mini Kit (Qiagen). RNA purity was verified by the 260 nm/280 nm 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\textsuperscript{®} Green JumpStart\textsuperscript{TM} Taq ReadyMix\textsuperscript{TM} (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 \(\beta\)-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).

**Analysis of cell death and apoptosis**

Cells were plated in flat bottomed 96-well plates and incubated for 24 hr until 70% confluent. Cells were treated with HXR9 or CXR9 at a range of dilutions for 2 hr. 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 hr 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°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 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 \(\pm 2\)-, 4- and 8-fold this concentration. Cell viability was then measured by the MTS assay (as described earlier) 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 and CI > 1 indicates 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 (Supporting Information 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 RNAlater\(^{\text{®}}\) and stored at \(-20^\circ\text{C}\) 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 \(\beta\)-actin. All sample and data collection received an ethical approval by the institutional ethics committee (MREC-09/H11103/50).

**Mouse in 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 hr 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 100 \(\mu\)L Hanks media (Sigma) containing \(10^6\) SKOV-3 cells in 50% matrigel (BD Bioscience). Once tumours reached a volume of approximately 100 mm\(^3\), 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 100 mg/kg HXR9 intratumorally (IT), with subsequent dosing of 10 mg/kg twice weekly. The cisplatin treatment group received a weekly dose of 3 mg/kg via intra-peritoneal 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

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 (Fig. 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 counterpart-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 (Fig. 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 (Fig. 3). The strongly overexpressed genes included HOXA9 (p = 1.86 × 10^{-10}), previously reported to be related to the HGS histotype,3 however, HOXA3 was expressed to a far higher level, (p = 9.55 × 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

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

| 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            |

Hazard ratios and Confidence intervals were calculated using the Log rank model.

---

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 scores transformation (genes) was used. Red colour for a gene indicates expression above the median and blue indicates expression below the median.
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. 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. This drug has been shown previously to induce apoptosis in cancer cells with highly dysregulated HOX expression profiles, including the ovarian cancer cell line SKOV-3. SKOV-3 is platinum-resistant, although its origin has recently been questioned.

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 renders inactive) have been described previously. 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). 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 (Figs. 4a and 4b). 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. 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 (Fig. 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.

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. 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. 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.
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 as ** and <0.001 as *** with respect to untreated cells. (b) Example flow cytometry plots for untreated; CXR9 25 μM; CXR9 50 μM; HXR9 25 μM and HXR9 50 μM treated SKOV-3 cells.
Figure 4. (Continued). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Tumor Markers and Signatures

expression in ovarian cancer has been reported previously in a
upregulation has been shown to be associated with the
HOXB4
lated, the most significant being
HOXB4
lated SC with 1
mor activity of HXR9 and cisplatin alone or in combination against
In vivo
Figure 5.

Although the function of the HOX genes in cancer remains
unclear, there have been reports that they act as tumour sup-
pressor genes or oncogenes. In ovarian cancer both HOXB7
and B13 expression has been linked to the invasive characteris-
tics of ovarian cancer cells,
25
and HOXB7 has been shown to be
a regulator of bFGF- a potent mitogenic and angiogenic fac-
tor
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 upregu-
lated, 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 can-
cer 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 altera-
tions and three members of the HOXB family, HOXB2, B5 and
B8 were among the focally amplified regions
33
further support-
ing a possible oncogenic role of HOXB genes in ovarian cancer
and emphasising the overlapping functions which exist be-
 tween 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 overexpres-
sion was common in two of the three cell lines (when com-
pared to the platinum-sensitive counterpart). These results
therefore demonstrate that HOXB genes are likely to play a
role in developing platinum resistance; although further study
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 differen-
tiation during development, but has been reported to be over-
expressed 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
35
and in vivo.
36
High-throughput micro-
array 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
HOX cluster has also been found in hepatocellular carcinoma
(HCC), in particular HOXA13.
37

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

HOXCI3 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 mye-
loid leukaemia (AML).
41
The HOXD1 gene appears to be
involved in cell differentiation,
42
whereas HOXD13 is deregu-
lated 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 neoplas-
tic transformation.
46

Determination of HOX gene dysregulation may be under-
taken routinely in the clinical setting using fresh or archived
patient tissue and such information could be used for strati-
ﬁng patients in terms of prognosis. Furthermore, we have
shown that our novel agent HXR9, a peptide capable of dis-
rupting HOX gene function by inhibiting HOX binding to its
cofactor, 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 ovar-
ian 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 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 approaches to achieve this is warranted.

Acknowledgment

This research was supported by GRACE, a gynaecological charity based in Surrey, UK.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012;62:10–29.
2. Naora H, Montz FJ, Chai CY, et al. Aberrant expression of homeobox gene HOXA7 is associated with Mullerian-like differentiation of epithelial ovarian tumors and the generation of a specific autologous antibody response. Proc Natl Acad Sci USA 2001;98:15299–14.
3. Cheng W, Liu J, Yoshida H, et al. Lineage infidelity of epithelial ovarian cancers is controlled by HOX genes that specify regional identity in the reproductive tract. Nat Med 2005;11:1531–7.
4. Taylor HS, VandenHeuvel GB, Igarashi P. A conserved HOx axis in the mouse and human female reproductive system: Late establishment and persistent adult expression of the HOX cluster genes. Biol Reprod 1997;57:1338–45.
5. Kelly ZL, Michael A, Butler-Manuel S, et al. HOX genes in ovarian cancer. J Ovarian Res 2011;4.
6. Calvo R, West J, Franklin W, et al. Altered HOX and WNT7A expression in human lung cancer. Proc Natl Acad Sci USA 2000;97:12776–81.
7. Miller GJ, Miller HL, van Bokhoven A, et al. Aberrant HOX expression accompanies the malignant phenotype in human prostate. Cancer Res 2003;63:5879–86.
8. Raman V, Martensen SA, Reisman D, et al. Compromised HOX4 function can limit p53 expression in human breast tumours. Nature 2000;405:974–8.
9. Vider BZ, Zimber A, Hirsch D, et al. Human colorectal carcinogenesis is associated with deregulation of homeobox gene expression. Biochem Biophys Res Commun 1997;232:742–8.
10. Tega Cgarn. Integrated genomic analyses of ovarian carcinoma. Nature 2011;474:4609–15.
11. Morgan R, Piraz PM, Shears L, et al. Antagonism of HOX/PBX dimer formation blocks the in vivo proliferation of melanoma. Cancer Res 2007;67:5806–13.
12. Ploswright L, Harrington KJ, Pandha HS, et al. HOX transcription factors are potential therapeutic targets in non-small-cell lung cancer (targeting HOX genes in lung cancer). Br J Cancer 2009;100:470–5.
13. Morgan R, Ploswright L, Harrington K, et al. Targeting HOX and PBX transcription factors in ovarian cancer. BMC Cancer 2010:10:89.
14. Shears L, Ploswright L, Harrington K, et al. Disrupting the Interaction Between HOX and PBX Causes Necrotic and Apoptotic Cell Death in the Renal Cancer Lines Calci-2 and 769-P. J Urol 2008;180:2196–201.
15. Morgan R, Boxall A, Harrington K, et al. Targeting the HOX/PBX dimer in breast cancer. Breast Cancer Res Treat 2012;136:389–98.
16. Domcke S, Sinha R, Levine DA, et al. Evaluating cell lines as tumour models by comparison of genomic profiles. Nat Commun 2013;4.
17. Stornach EA, Alfradat A, Rama N, et al. HDAC4-Regulated STAT1 Activation Mediates Platinum Resistance in Ovarian Cancer. Cancer Res 2011;71:4412–22.
18. Heid CA, Stevens J, Livak KJ, et al. Real time quantitative PCR. Genome Res 1996;6:986–94.
19. Chou T-C. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006;58:621–81.
20. Workman P TP, BALKWELL F, BALMAIN A, CHAPLIN D, Double J, EMBLETION J, NEWELL D, RAYMOND R, STABLES J, STEPHENS T, WALLACE J. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia. 1998.
21. Langdon SP, Lowrie SS, Hay FF, et al. Characterization and Properties of Nine Human Ovarian Adenocarcinoma Cell Lines. Cancer Res 1988;48:6166–72.
22. Crijns APG, de Graeff P, Thompson A, et al. HOX genes: seductive science, mysterious mechanisms. The Ulster Medical Journal 2006;75:23–31.
23. Ota T, GIKS CB, LONGACRE T, et al. HOXA7 in epithelial ovarian cancer: Interrelationships between differentiation and clinical features. Reprod Sci 2007;14:605–14.
24. Yamashita T, Tazawa S, Yawei Z, et al. Suppression of invasive characteristics by antisense introduction of overexpressed HOX genes in ovarian cancer cells. Int J Oncol 2000;26:931–8.
25. Naora H, Yang Y, Montz FJ, et al. A serologically conserved HOX axis in the mouse and human female reproductive system: Late establishment and persistent adult expression of the HOX cluster genes. Biol Reprod 1997;57:1338–45.
26. Naora H, Yang Y, Montz FJ, et al. A serologically conserved HOX axis in the mouse and human female reproductive system: Late establishment and persistent adult expression of the HOX cluster genes. Biol Reprod 1997;57:1338–45.
27. Rubin E, Wu XY, Zhu T, et al. A role for the HOXB7 homeodomain protein in DNA repair. Proc Natl Acad Sci U S A 2001;98:4060–5.
28. Rubini E, Wu XY, Zhu T, et al. A role for the HOXB7 homeodomain protein in DNA repair. Cancer Res 2007;67:1527–35.
29. Miao J, Wang Z, Provencher H, et al. HOXB13 promotes ovarian cancer progression. Proc Natl Acad Sci U S A 2007;104:17093–8.
30. Hong HH, Lee JK, Park JJ, et al. Expression pattern of the class I homeobox genes in ovarian carcinoma. J Gynaecol Oncol 2010;21:29–37.
31. Bodey B, Siegel SE, Kaiser HE. Immunocytochemical detection of the homeobox B3, B4, and C6 gene products in breast carcinomas. Anti-cancer Res 2000;20:3281–6.
32. Domcke S, Sinha R, Levine DA, et al. Evaluating cell lines as tumour models by comparison of genomic profiles. Nat Commun 2013;4.
33. Stornach EA, Alfradat A, Rama N, et al. HDAC4-Regulated STAT1 Activation Mediates Platinum Resistance in Ovarian Cancer. Cancer Res 2011;71:4412–22.
34. Heid CA, Stevens J, Livak KJ, et al. Real time quantitative PCR. Genome Res 1996;6:986–94.
35. Chou T-C. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006;58:621–81.
36. Workman P TP, BALKWELL F, BALMAIN A, CHAPLIN D, Double J, EMBLETION J, NEWELL D, RAYMOND R, STABLES J, STEPHENS T, WALLACE J. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia. 1998.
37. Langdon SP, Lowrie SS, Hay FF, et al. Characterization and Properties of Nine Human Ovarian Adenocarcinoma Cell Lines. Cancer Res 1988;48:6166–72.
38. Crijns APG, de Graeff P, Thompson A, et al. HOX genes: seductive science, mysterious mechanisms. The Ulster Medical Journal 2006;75:23–31.
39. Ota T, GIKS CB, LONGACRE T, et al. HOXA7 in epithelial ovarian cancer: Interrelationships between differentiation and clinical features. Reprod Sci 2007;14:605–14.
40. Yamashita T, Tazawa S, Yawei Z, et al. Suppression of invasive characteristics by antisense introduction of overexpressed HOX genes in ovarian cancer cells. Int J Oncol 2000;26:931–8.
41. Naora H, Yang Y, Montz FJ, et al. A serologically conserved HOX axis in the mouse and human female reproductive system: Late establishment and persistent adult expression of the HOX cluster genes. Biol Reprod 1997;57:1338–45.