

## Expression of a homeobox gene (SIX5) in borderline ovarian tumours

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### Abstract

**Aims**—To assess the expression of SIX5 (a homeobox gene) mRNA in surface coelomic epithelium, endocervical epithelium, Fallopian tube epithelium, and benign, borderline, and malignant epithelial ovarian tumours.

**Methods**—10 normal premenopausal ovaries, 10 normal Fallopian tubes, 10 normal cervixes, 10 normal postmenopausal ovaries, 10 benign epithelial ovarian tumours, 10 malignant epithelial ovarian tumours, and 40 borderline epithelial ovarian tumours were studied retrospectively. The tissues had been fixed in formalin and embedded in paraffin wax. The tumours had previously been typed into mucinous, serous, or mixed tumours and assigned to the borderline category according to the FIGO/WHO criteria. Expression was assessed by in situ binding of SIX5 specific sense and antisense riboprobes. Hybridisation of the riboprobes was detected using a standard immunohistochemical technique and the results correlated with expression in the normal epithelium of the endocervix, Fallopian tube, surface coelomic epithelium, and ovarian tumours.

**Results**—Expression of SIX5 mRNA was demonstrated in normal Fallopian tube epithelium and normal endocervical epithelium. SIX5 mRNA was not detected in normal ovarian epithelial tissue at any of the times studied during the menstrual cycle. Expression of SIX5 was not shown in benign epithelial ovarian tumours or in any of the malignant epithelial ovarian tumours. In 31 of 37 borderline epithelial ovarian tumours (84%), SIX5 expression was found in the epithelial cells.

**Conclusions**—SIX5 expression is present in the normal epithelium throughout most of the female reproductive tract, suggesting it may have a role in maintaining epithelial differentiation in these tissues. SIX5 expression appears to be restricted to borderline epithelial ovarian tumours and may be a marker of epithelial differentiation in these tumours; thus borderline ovarian tumours may not be part of a continuum of disease between benign and malignant epithelial ovarian tumours. Further investigation of expression of SIX5 may clarify the molecular processes that promote differentiation of the ovarian surface epithelium.

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There is considerable debate about the natural history of the development of malignant epithelial ovarian tumours. This arises because of difficulties in accessing ovarian tissue and because of the poor correlation between clinical evaluation at laparoscopy and histology,<sup>1-4</sup> and between ultrasound appearances and histology. It is well established that early stage ovarian cancer has a much better five year survival than advanced presentation disease (80% *v* 35%), but the difficulty lies in the detection of early malignant or premalignant changes in the ovary. It has been suggested that borderline epithelial ovarian tumours are part of a continuum of progressive change from benign to malignant,<sup>5</sup> showing some features of malignancy in the epithelial cells but without stromal invasion (as defined by the FIGO/WHO criteria).<sup>6,7</sup> The epidemiological evidence suggests a link between borderline and malignant epithelial ovarian tumours, with age at first childbirth, parity, contraceptive pill use, and postmenopausal status being correlated with the development of both types of tumour.<sup>8</sup> The incidence of both tumour types is increasing, although borderline epithelial ovarian tumours remain much less prevalent than malignant epithelial ovarian tumours, with incidences of 4.8/100 000 person-years and 14/100 000 person-years, respectively, in Norway.<sup>9</sup>

Detailed knowledge of the pathogenesis of ovarian tumours, and in particular the timing of progression of the disease, would help the detection of early stage disease and optimise the timing of surgical or chemotherapeutic interventions. Advances in molecular techniques have allowed us to determine genetic variations between different types of tumour and the sequences of molecular events that lead to malignant change. In the case of the ovary there is conflicting molecular evidence about the genesis of malignant epithelial ovarian tumours and where or whether borderline epithelial ovarian tumours fit in. Studies of ovarian tumours have concentrated on molecular changes known to occur in other malignancies such as those affecting oncogenes, tumour suppressor genes, and programmed cell death signalling such as p53. The differentiation from totipotent to specialised epithelial cells is common to benign, borderline, and malignant epithelial ovarian tumours and suggests that genes important in initiating patterned differentiation may be a molecular trigger common to the range of epithelial ovarian tumours. Homeobox genes are good candidates for triggering the molecular changes that may be involved in patterns of differentiation

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and proliferation in embryonic tissues and in maintaining patterning and differentiation in adult tissues. Recent studies have shown homeobox gene involvement in leukaemias<sup>10</sup> in solid tumours such as breast,<sup>11</sup> kidney, lung, and colon.<sup>12</sup>

The aim of this study was to determine the expression of a homeobox gene (SIX5) in the ovary in surface coelomic epithelium, benign epithelial ovarian tumours, borderline epithe-

lium, and malignant epithelium. It was hoped that by analysing changes in the expression in the different epithelia, the relation between the different types of ovarian tumour could be assessed and any association between them defined.

## Methods

### TISSUE SAMPLES

Ten normal premenopausal ovaries, 10 normal postmenopausal ovaries, 10 benign ovarian tumours, 37 borderline epithelial ovarian tumours, and 10 malignant epithelial ovarian tumours were studied retrospectively. The normal ovaries were collected at different stages throughout the menstrual cycle, as ascertained by the date of the last menstrual period (LMP). Normal ovarian tissue was obtained from women undergoing hysterectomy for gynaecological procedures unrelated to potential ovarian pathology. The benign ovarian tumours were obtained from women undergoing oophorectomy at the time of hysterectomy and undergoing oophorectomy for tissue diagnosis of an ovarian mass. Five of the cysts studied were inclusion cysts with serous epithelium close to the coelomic surface of the ovary. Two were serous cystadenomas and three were mucinous cystadenomas. The borderline tumours were assessed according to the WHO/Figo criteria<sup>6,7</sup> and represented serous, mucinous, and mixed serous and mucinous types, stage 1A–1C. Only blocks from the tumours themselves (and not implants) were used for the study. Nineteen (51%) were mucinous cystadenomas, 16 (43%) were serous cystadenomas, and two (5.4%) showed features of both serous and mucinous cystadenomas. Five serous cystadenocarcinomas FIGO stage III and five mucinous cystadenocarcinomas FIGO stage III were studied. All the tissues had been fixed in formalin and embedded in paraffin wax. The ovaries were examined after staining with haematoxylin and eosin. Only ovaries that had intact surface coelomic epithelium over the ovarian surface were used. The fragile nature of the epithelium meant that many specimens were denuded of epithelium during handling and preparation and therefore were not suitable for analysis. Control sections of formalin fixed, wax embedded tissue from normal Fallopian tube and normal cervix were analysed to ascertain whether serous and mucinous differentiated cells in these tissues showed expression of SIX5.

The normal ovarian tissue, normal cervical tissue, Fallopian tubes, benign epithelial ovarian tumours, malignant epithelial ovarian tumours, and 21 borderline epithelial ovarian tumours were obtained from the archive of the pathology laboratory, Western Infirmary, Glasgow, and 16 borderline epithelial ovarian tumours were obtained from the pathology laboratory, Glasgow Royal Infirmary.

### IN SITU HYBRIDISATION

Sections were cut at 4  $\mu$ m thickness from paraffin blocks and mounted onto APES coated slides. The sections were processed according to a standard protocol<sup>13</sup> and

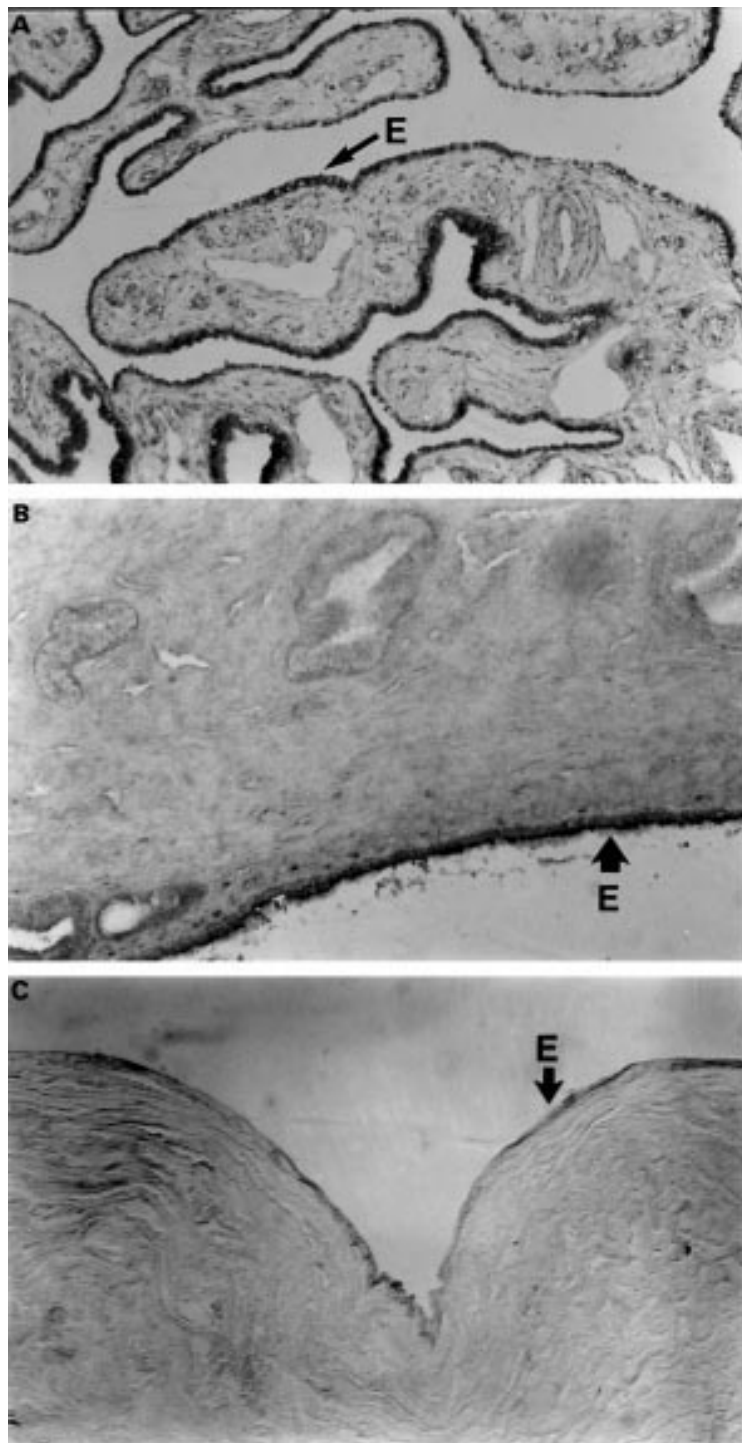


Figure 1 Analysis of SIX5 expression in (A) normal Fallopian tube showing strong expression in the epithelium ( $\times 65$ ); (B) normal endocervix demonstrating strong expression in the endocervical epithelium, but not in the deeper glandular epithelium ( $\times 65$ ); (C) normal ovary ( $\times 65$ , oblique illumination) showing no staining in the epithelium. Epithelial layers are marked E.

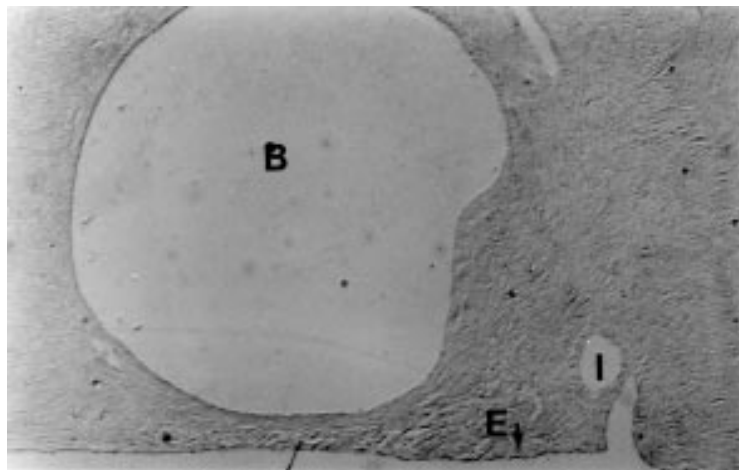


Figure 2 Analysis of coelomic surface epithelium (E), inclusion cyst epithelium (I), and benign cyst epithelium (B) ( $\times 33$ ). None of the epithelia show expression of SIX5.

hybridised with digoxigenin (Boehringer Mannheim) labelled sense and antisense SIX5 riboprobes. Digoxigenin labelled sense and antisense SIX5 riboprobes were generated by *in vitro* transcription of pSIX5.1. The SIX5 riboprobe generated was designed to specifically detect only SIX5 mRNA, as it contains sequence mainly from the 5'UTR and does not include any regions that are similar to other human SIX genes.

The SIX5 subclone, pSIX5.1, was generated by first amplifying genomic DNA using a forward primer in the 3'UTR of DMPK, FAS (5' TCC TCA CTG CGC TGC TCT C 3') and a reverse primer in exon A of SIX5, RAS (5' TGC GCA GTC GAT ACT TGT CCA C 3'). The PCR product was subcloned into the T vector pMOSBlue (Amersham International) and then cut with EcoRI-BamHI to release a SIX5 fragment of 652 base pairs (bp) (662–1314 of accession number X84813). The 652 bp SIX5 fragment was ligated unidirectionally into EcoRI-BamHI cut pBluescript<sup>®</sup> SK(+), to create pSIX5.1. The SIX5 sense riboprobe was generated by linearising pSIX5.1 with BamHI and transcribing with T7 RNA polymerase (Promega), and the SIX5 antisense riboprobe was generated by linearising pSIX5.1 with EcoRI and transcribing with T3 RNA polymerase (Promega).

One microgram of each template was linearised and the DNA transcribed with 40 units of T7 or T3 RNA polymerase (Promega) at 3°C for four hours. The reaction mix contained 10 mM dithiothreitol (DTT), 20

units of RNasin (RNase inhibitor) (Promega), 1  $\times$  transcription buffer (Promega), 1 mM of adenosine triphosphate (ATP), 1 mM of cytosine triphosphate (CTP), 1 mM of guanosine triphosphate (GTP), 0.65 mM of uridine triphosphate (UTP), and 0.35 mM digoxigenin-11-UTP (Boehringer Mannheim). The riboprobes were precipitated and resuspended in 40  $\mu$ l of DEPC treated water.

The probe sequence was checked by comparing it with the sequence of all known human SIX genes and all sequences in the DNA and protein databases. It did not match any sequence apart from SIX5. The specificity of the riboprobe interaction was checked for *in situ* hybridisation experiments of other human tissue.<sup>15</sup> The specificity of the staining was checked by RNase treatment before and after hybridisation with the riboprobe and confirmed that the SIX5 antisense riboprobe was specifically interacting with mRNA.

In control experiments, sections were treated with 100 mg/ml RNase A at 37°C for 30 minutes before the prehybridisation step or for 30 minutes after the hybridisation step; with hybridisation buffer without riboprobe; or by hybridisation with sense riboprobe.

The interactions were detected colorimetrically (blue/black) with alkaline phosphatase substrates (0.33 mg/ml NBT (nitroblue tetrazolium salt in 70% vol/vol dimethyl formamide) (Sigma), 0.17 mg/ml BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium salt in 100% dimethyl formamide) (Sigma), and 0.23 mg/ml levamisole (Sigma) at room temperature.

#### PHOTOMICROGRAPHS

Photomicrographs were taken of the sections on an Olympus BX 50 microscope on Kodak ectochrome 64T film. Oblique illumination was produced by introducing a flat opaque object into the condenser.

#### Results

SIX5 expression was demonstrated in normal surface endocervical epithelium and normal Fallopian tube epithelium, as demonstrated in fig 1. Staining was strong and homogeneous within the epithelia of these tissues. The glandular epithelium of the cervix did not appear to express SIX5.

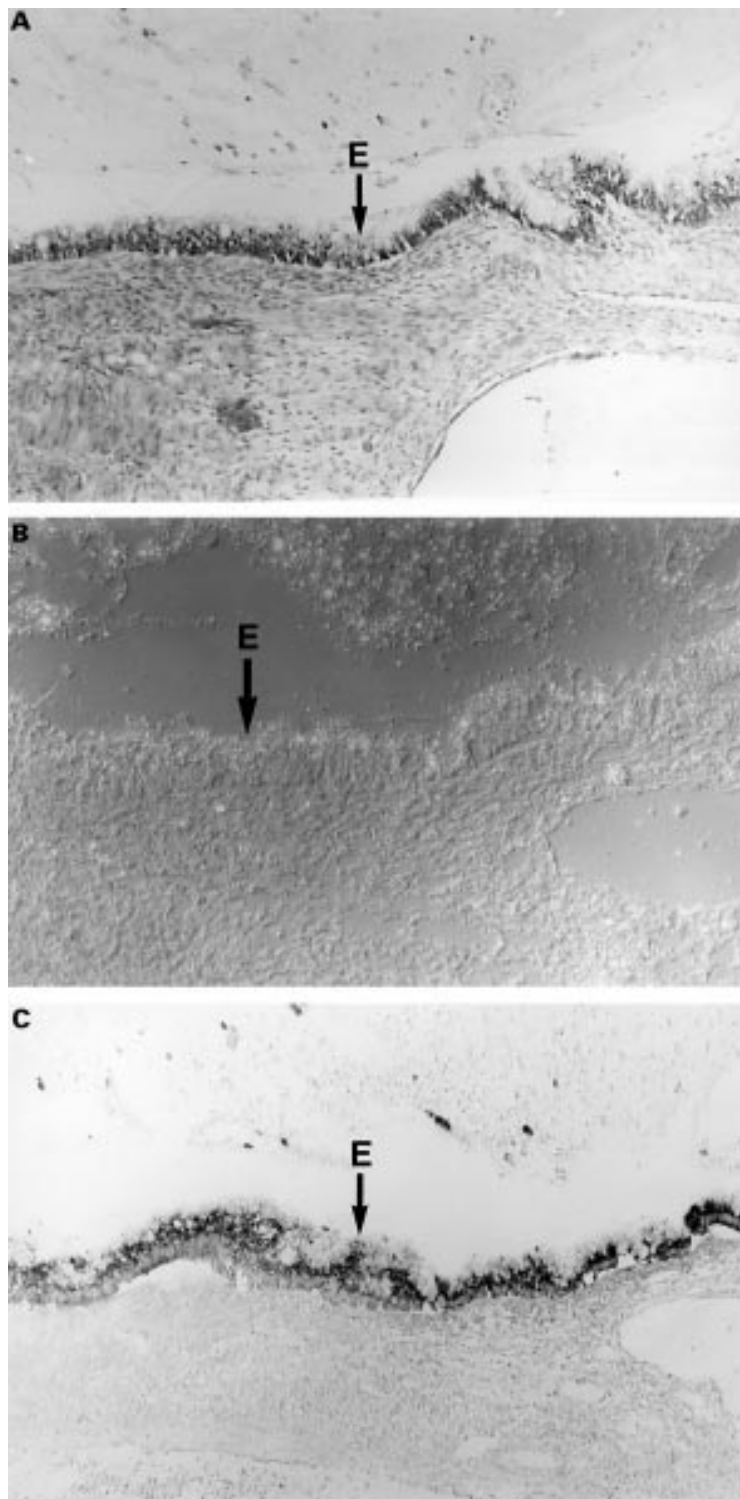
The 10 normal premenopausal ovaries were obtained at various stages of the menstrual cycle. Four were collected in the early follicular phase, two at mid cycle, and four in the secretory phase according to LMP dates. Expression of SIX5 was not demonstrated, either in normal premenopausal ovarian epithelial tissue (as shown in fig 1) at any time during the menstrual cycle sampled in our study, or in postmenopausal ovarian epithelial tissue. Expression of SIX5 was not shown in benign epithelial ovarian tumours or inclusion cyst epithelium (fig 2) or in any of the malignant epithelial ovarian tumours.

In 31 of 37 borderline tumours (85%), focal SIX5 expression was found in the epithelial cells. The distribution and intensity of the staining did not correlate with morphological

Table 1 Expression of SIX5 mRNA in ovarian epithelium

Tissue type	SIX5 expression	
	Positive	Negative
Normal follicular phase ovary	0	4
Normal midcycle ovary	0	2
Normal secretory phase ovary	0	4
Normal postmenopausal ovary	0	10
Malignant EOTs	0	10
Borderline serous EOTs	16 (13 stage 1A; 2 stage 1B; 1 stage 1C)	0
Borderline mucinous EOTs	14 (stage 1A)	5 (stage 1A)
Borderline mixed serous and mucinous EOTs	1 (stage 1A)	1 (stage 1A)

EOT, epithelial ovarian tumour.



**Figure 3** Analysis of *SIX5* mRNA expression in serial sections of a borderline epithelial ovarian tumour. (A) Stained with haematoxylin and eosin ( $\times 100$ ); (B) hybridised with sense *SIX5* riboprobe (control) ( $\times 100$  oblique illumination); (C) hybridised with antisense *SIX5* riboprobe ( $\times 100$  oblique illumination) showing expression in the epithelial cell layer. Epithelial layers are marked E.

changes in the epithelial cells in different regions of the borderline tumours. Control sections hybridised with sense riboprobe showed less or no staining in most cases. In 10 cases, staining with the sense riboprobe was as intense as that seen with the antisense probe. Treatment with RNase A before hybridisation decreased staining in all the antisense positive

tissues, while treatment after hybridisation failed to alter the intensity of staining, indicating that the antisense riboprobe was specifically binding to *SIX5* mRNA. All the positive borderline epithelial ovarian tumours showed long stretches of epithelium, with intense staining in the perinuclear region and cytoplasm of the cells. A summary of these results is shown in table 1 and examples of expression of *SIX5* and the sense riboprobe control are shown in fig 2. There was variation in distribution and intensity of staining in different areas of the epithelium as demonstrated in fig 3. The same pattern of variation was seen in labelled serial sections suggesting that this reflected a true difference in expression and was not an artefact of the labelling procedure.

### Discussion

The aim of molecular studies in ovarian epithelial disease is to develop a detailed understanding of tumour progression akin to the paradigm of bowel cancer. The discovery of mutations in *BRCA1* and *BRCA2* genes has allowed identification of some individuals at high risk with a strong family history of ovarian epithelial cancer, but there are no published data showing an increase in both borderline and malignant epithelial ovarian tumours which would lend support to a common molecular pathway in their genesis.

Changes in microsatellite repeat number have been shown in borderline and early ovarian tumours and some advanced tumours.<sup>14-16</sup> In individual women with mismatch repair gene mutations (which lead to widespread microsatellite instability), a 10% incidence of malignant epithelial ovarian tumours can be anticipated.<sup>16</sup> *SIX5* is a homeobox gene situated adjacent to a well known site of repeat instability, a (CTG)<sub>n</sub> repeat on chromosome 19.<sup>17</sup> Expansion of this (CTG)<sub>n</sub> repeat is associated with the development of myotonic dystrophy. A change in repeat number at the DM locus has been found in breast tumours.<sup>18</sup> In a previous study we have not observed (CTG)<sub>n</sub> repeat expansion at this site in DNA extracted from a variety of benign and malignant ovarian tumours compared with normal tissue DNA (Thomas M, unpublished data). *SIX5* is a member of the *SIX* gene family and encodes a homeodomain transcription factor protein capable of altering gene expression at other sites in the genome. All the *SIX* genes are characterised by two functionally important regions, the *SIX* box and the homeobox, from which the name was derived. Reverse transcriptase polymerase chain reaction (RT-PCR) and northern blot analyses have shown widespread expression of *SIX5* in fetal and adult tissues.<sup>17</sup> Localisation of *SIX5* mRNA to specific cell types has only been characterised in the eye, where *SIX5* was shown to be expressed in epithelial cells of various eye structures.<sup>13</sup>

Previous work on ovarian pathology has not shown consistent sequential changes in the genome analogous to those seen in bowel tumours. Molecular changes have been described in both borderline epithelial ovarian

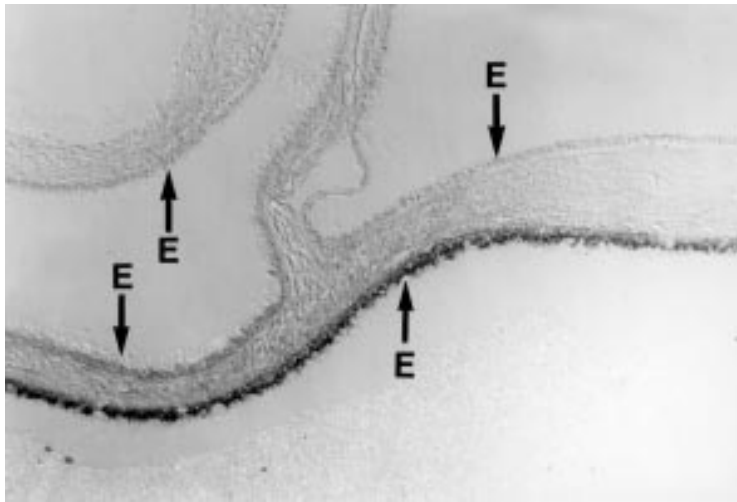


Figure 4 Variation in *SIX5* expression in a stage IA serous borderline epithelial ovarian tumour, showing the focal nature of expression in the epithelium of some cysts but not others ( $\times 65$ , oblique illumination).

tumours and malignant epithelial ovarian tumours in K-ras oncogenes,<sup>19, 20</sup> p53 expression<sup>21</sup>, c-erb B-2 expression,<sup>22</sup> loss of heterozygosity at various loci, and changes in microsatellite repeat number.<sup>14-16</sup>

These conflicting data suggest that either the clonal molecular event in the genesis of ovarian tumours has yet to be defined, or a range of molecular events unique to each tumour may trigger malignant change, or the suggestion that borderline epithelial ovarian tumours are a premalignant stage of ovarian tumorigenesis is incorrect. In order to further our understanding of the events involved in tumorigenesis, we have investigated the expression of *SIX5*, which we have shown in the epithelium of the normal female reproductive tract. Our study showed that most (85%) borderline epithelial ovarian tumours show expression of *SIX5* in their epithelium. In situ hybridisation techniques have allowed the investigation of gene activation in whole tissues, although the use of sense riboprobes as a control is notoriously inconsistent and makes interpretation difficult in some cases. In the 10 cases where staining with the sense and antisense probes was equally intense, pretreatment with RNase decreased the staining of the sense but not the antisense probe, indicating some weak binding which could be disrupted with RNase. Another possible explanation for these findings might be that *SIX5* is bidirectionally transcribed, although *SIX5* is a fairly well documented gene and there has been no evidence to date to support this.

The focal nature of the staining could have been an artefact of processing and staining the tissue. However, this is not likely as analysis of serial sections showed the same distribution of focal staining in adjacent tissues, and staining of normal epithelia in Fallopian tube was homogeneous throughout all the epithelial cells. The surface endocervical epithelium appeared to be expressing *SIX5*, although the deeper glandular epithelium did not. This phenomenon was present in all the cervixes studied and although the possibility of an edge

artefact remains, this was not seen in borderline tumours such as in fig 4, where some edges were clearly positive and others negative. Further work on microdissected regions showing focal and no staining of the mRNA message would determine whether there is a quantitative change associated with these observations. The lack of hybridisation in the surface coelomic epithelium and malignant tumour samples does not mean that there is no expression of *SIX5*. In situ hybridisation may not be sufficiently sensitive to detect the small amounts of mRNA production that may be required to maintain patterning in an already differentiated tissue. Indeed, RT-PCR studies on normal whole ovary have shown *SIX5* mRNA expression, although it is not possible to say which cell types within the ovary were contributing to the mRNA detected (Winchester C, unpublished data).

There are two different types of ovarian mucinous tumour of borderline malignancy. In most cases, the mucinous epithelium is of intestinal type, but in a minority it is of endocervical type.<sup>23, 24</sup> It has been shown that this morphology correlates with differences in amylase production in the tumour cells and these differences are also observed in normal bowel and endocervical epithelium.<sup>25</sup> All the mucinous borderline epithelial ovarian tumours in this study were morphologically classified as intestinal epithelial type and were stage IA. If the role of *SIX5* was confined to the female genital tract, it may have been anticipated that only serous tumours would show expression of *SIX5*. This was not the case, as *SIX5* expression was present in both serous and mucinous borderline epithelial ovarian tumours (although all the borderline tumours that failed to show expression were of the mucinous type).

The lack of expression in the epithelium of premenopausal ovaries at various stages in the menstrual cycle, in postmenopausal ovaries, and in benign and malignant epithelial ovarian tumours suggests that borderline epithelial ovarian tumours are not part of a continuum of disease between benign and malignant tumours. However, our findings do not rule out this possibility. Transient overexpression of homeobox genes in bowel epithelium has suggested a regulatory link between the *HOXB8* and several tumour suppressor genes such as *DCC*, *APC*, and *TGF $\beta$* .<sup>26</sup> Overexpression of another *SIX* gene (*SIX1*) has been found in breast tumours in association with abrogation of the G2 cell cycle check point. This is of particular interest as the epidemiology of and the genetic (*BRCA1* and *BRCA2*) predisposition to breast and ovarian cancer are very similar. It is possible that the *SIX* genes may have a regulatory link with *BRCA1* and *BRCA2*.

The expression of *SIX5* in borderline epithelial ovarian tumours shows that a molecular change has occurred in differentiated epithelial cells and suggests its potential as a molecular trigger of pathogenesis in most borderline epithelial ovarian tumours. Further investigation by microdissection studies of adjacent and contralateral benign, borderline,

and malignant epithelial ovarian tumours and determination of the links with genes involved in cell proliferation are needed to clarify the relation between benign, borderline, and malignant ovarian epithelial change.

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