Molecular detection of tumour DNA in serum and peritoneal fluid from ovarian cancer patients

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Summary We have analysed DNA extracted from the serum and peritoneal fluid of 20 ovarian cancer patients for the presence of tumour-specific genetic alterations. The 20 patients included six with stage Ia disease. Using six polymorphic microsatellite loci we were able to detect novel alleles or loss of heterozygosity in 17/20 serum samples and 12/19 peritoneal fluid samples. Tumour-specific abnormalities were detected in the serum of all but one of the stage Ia cases. Half of the occurrences of loss of heterozygosity identified in primary tumour material were detectable in the serum samples. Novel alleles indicative of microsatellite instability were found in 3/6 patients with stage Ia disease but in only 1/14 of patients with more advanced disease. One of the eight patients in the control group displayed abnormalities in her serum DNA. The ease with which tumour-specific alterations were detected in serum and peritoneal samples from ovarian cancer patients, using a panel of only six polymorphic microsatellite markers on four chromosomes, suggests that molecular detection methods could prove useful in the staging, monitoring and screening of this disease.

Keywords: ovarian cancer; molecular detection; microsatellite instability; serum

Ovarian cancer is the leading cause of death from gynaecological malignancy in the UK, with over 4000 women dying annually. The high mortality is attributable to the lack of effective screening strategies and the fact that early-stage disease remains clinically silent. Most patients present with advanced disease, for which the long-term survival remains <30%. However, if early stage disease could be detected the 5-year survival could rise to 80–95% (Berek, 1994). Current ovarian cancer screening methods using serum tumour markers and ultrasound/Doppler techniques lack the specificity to achieve this goal at present.

Nucleic acid-based molecular detection methods have been used to demonstrate the presence of tumour cells in samples of stool (Sidransky et al, 1992), urine (Mao et al, 1996), sputum (Mao et al, 1994) and blood (Smith et al, 1991). In addition, the same approaches have confirmed the presence of free circulating tumour DNA in the serum of patients with epithelial tumours of the lung (Chen et al, 1996), head and neck (Nawroz et al, 1996) and colon (Hibi et al, 1998). The majority of these studies have demonstrated clear clonality between the genetic alterations observed in test samples and the corresponding primary tumour material. The types of alteration recorded have included specific gene mutations, loss of heterozygosity (LOH) and microsatellite instability (MI).

Only 5–10% of ovarian carcinomas appear to involve a familial predisposition. The two major syndromes involved are the familial breast and ovarian cancer syndrome, associated with inherited mutations in the BRCA1 gene, and the Lynch II syndrome (HNPPC), associated with inherited defects in the DNA mismatch repair system. Abnormalities associated with sporadic forms of the disease include mutations in k-ras and p53 genes, and changes in expression of HER-2 (Gallion et al, 1995). Allelotyping studies have identified common sites of LOH on chromosomes 5q, 6q, 11p, 13q, 14q, 15q, 18q, Xp (Cliby et al, 1993; Osborne and Leech, 1994), and particularly on chromosome 7q (Zenklusen et al, 1995), 9q (Schultz et al, 1995) and 17p (Phillips et al, 1993). The occurrence of novel alleles at microsatellite loci, due to either a replication error phenotype (RER*) resulting from defective mismatch repair or general genetic instability, has been described in 17–37% of ovarian tumours (Fujita et al, 1995; King et al, 1995; Sood and Buller, 1996). However, MI may occur in up to 75% of stage I ovarian tumours, and 71% of uncommon histopathological types such as endometrioid or mixed serous and mucinous tumours (King et al, 1995).

We obtained serum and peritoneal fluid from 20 women with epithelial ovarian carcinoma, and used a panel of six polymorphic microsatellite markers to detect LOH or novel alleles in the DNA extracted from the samples. The same analysis was carried out on tumour tissue microdissected from paraffin sections of the primary tumour from each case. The aim of this study was to establish whether tumour-specific biomarkers could be detected in the serum and peritoneal fluid of ovarian cancer patients, particularly those with early-stage disease.

MATERIALS AND METHODS

Sample collection

Samples were collected from 20 women undergoing laparotomy for ovarian cancer, and eight women with benign or physiological cysts. None of the women were known to carry a familial
predisposition for ovarian cancer, although one woman had a first-degree relative with breast cancer. The cancer cases included six stage Ia and 14 stage II–IV cases. In all six of the stage Ia cases total abdominal hysterectomy, bilateral salpingo-oopherectomy, omentectomy and peritoneal washings for cytology were taken, while ipsilateral pelvic node resection and para-aortic node sampling was carried out in three of the six. The pathological staging, serum CA125 and cytology results are shown in Table 1.

**DNA extraction**

**Normal DNA**

Ten millilitres of blood were taken into heparinized tubes, and 1 ml was used for extraction of white blood cell DNA using Nucleon (Scotlab).

**Serum DNA**

Ten millilitres of blood was taken, allowed to clot and then centrifuged at 1300 g. The serum supernatant was frozen at –80°C in aliquots and DNA was extracted from 400 μl using DNAzol (Gibco BRL).

**Peritoneal fluid DNA**

At laparotomy, 20 ml of ascitic fluid or pelvic peritoneal washings were taken, of which 10 ml were sent for routine cytological analysis, and the other 10 ml for molecular analysis. The latter samples were centrifuged at 1300 g to pellet any cells, which were washed twice in phosphate-buffered saline (PBS). The cells were frozen at –80°C in tissue culture-freezing medium (2.75 ml Dulbecco’s modified Eagle’s medium, 1.25 ml fetal calf serum, 1 ml dimethyl sulphoxide (DMSO)) in 1 ml aliquots. Cells were spun out of thawed aliquots and washed twice with 5 ml PBS to remove DMSO. Epithelial cells were then harvested by immuno-magnetic separation using Dynabeads coated with the epithelial specific antibody Ber EP4 (Dynal UK). DNA was extracted from the cells using Dynabeads DNA Direct (Dynal UK). A peritoneal fluid sample from patient 065 was unavailable for molecular analysis.

**Tumour DNA**

Areas of tumour tissue were microdissected from ten paraffin sections of primary tumours and DNA extracted using Nucleon (Scotlab). The precipitated DNA was resuspended in 50 μl of water.

**Cyst wall DNA**

For cases of benign and physiological cyst, areas corresponding to the cyst wall were microdissected and processed in the same way as the tumour DNA.

**Microsatellite analysis**

Polymerase chain reaction (PCR) amplification was carried out using oligonucleotide amplimers specific for six polymorphic DNA microsatellite loci:

- DP1 (D5S346) on 5q (Sprio et al, 1993)
- 486 (D7S486) on 7q (Gyapay et al, 1994)
- 522 (D7S522) on 7q (Gyapay et al, 1994)
- D11 (D11S904) on 11p (Gyapay et al, 1994)
- p53V (intron1 in p53) on 17p (Cawkwell et al, 1994)
- BRCA1 (D17S855) on 17q (Gao et al, 1995).

The chosen markers map to regions which commonly display LOH in ovarian carcinomas. One amplimer from each pair was fluorescently labelled using the dye-amidite method (Cawkwell et al, 1994).

The PCR reactions were set up in 25 μl of 1 × SuperTaq Reaction Buffer (HT Biotechnology Ltd) containing 200 μM dNTPs, 12.5 pmol of each amplifier, 1.5–6.0 mM magnesium

| Patient | Stage | Histology type and differentiation | Cytology | Tumour LOH | Serum LOH | Peritoneal fluid LOH |
|---------|-------|-----------------------------------|----------|------------|-----------|---------------------|
| 112     | Ia    | Endometrioid, Grade 1             | –        | 1/6        | –         | 0/6                 |
| 200     | Ia    | Endometrioid and mucinous, Grade 2| –        | 1/5        | –         | 0/5                 |
| 3775    | Ia    | Mucinous, Grade 2                 | –        | 0/1        | 5         | 0/4                 |
| 4224    | Ia    | Mucinous, Grade 2                 | –        | 0/3        | 2         | 0/3                 |
| 5475    | Ia    | Serous, Grade 2                   | –        | 3/4        | –         | 0/4                 |
| 065     | Ia    | Endometrioid, Grade 1             | –        | 1/3        | 1         | NA                  |
| 705     | Iic   | Serous, Grade 3                   | +        | 1/5        | –         | 1/4                 |
| 488     | Iic   | Papillary serous, Grade 2         | –        | 3/3        | –         | 1/3                 |
| 165     | Iic   | Papillary serous, Grade 2         | –        | 4/5        | –         | 1/5                 |
| 231     | Iic   | Serous, Grade 3                   | –        | 5/5        | 1         | 0/5                 |
| 042     | Iic   | Papillary serous, Grade 3         | –        | 3/4        | –         | 1/4                 |
| 577     | Iic   | Endometrioid & serous, Grade 3    | +        | 0/4        | –         | 1/4                 |
| 0776    | Iic   | Papillary serous, Grade 3         | +        | 0/3        | –         | 0/3                 |
| 9930    | Iic   | Serous, Grade 3                   | –        | 5/5        | –         | 5/5                 |
| 4992    | Iic   | Papillary serous, Grade 3         | –        | 2/4        | –         | 1/4                 |
| 5042    | Iic   | Endometrioid, Grade 3             | +        | 2/5        | –         | 0/5                 |
| 3944    | Iic   | Serous, Grade 3                   | –        | 0/4        | –         | 0/4                 |
| 4571    | Iic   | Papillary serous, Grade 3         | +        | 3/4        | –         | 2/3                 |
| 421     | Ic    | Endometrioid, Grade 3             | +        | 3/4        | –         | 3/4                 |
| Total   |       |                                    |          | 39/80      | 9         | 18/78               |

* FIGO staging of primary tumour; *presence of malignant cells in ascitic fluid or peritoneal washings; *proportion of informative (heterozygous) markers displaying loss of heterozygosity (LOH); *microsatellite instability. NA, not available for molecular analysis.
chloride (optimized for each pair of amplimers), and 1 μl of DNA. After denaturing at 95°C for 3 min and a pause at 80°C to add 0.5 U of SuperTaq polymerase (HT Biotechnology Ltd), the DNA was amplified using a programme of 92°C for 30 s, 56°C for 1 min, 72°C for 30 s, for a total of 35 cycles. The amplification products were visualized by agarose gel electrophoresis to estimate yield, electrophoresed on an ABI 373A DNA Sequencer (Applied Biosystems), and analysed using Genescan Analysis Software (Applied Biosystems), which determines the size of the PCR products and the amount of fluorescent signal. Suitable dilutions of PCR products were run to ensure that band intensities fell within the linear portion of the fluorescence detection range (500–4000).

Optimizing template DNA for PCR
We found that significantly different allele ratios could be obtained in our PCR reactions with different amounts of starting template DNA. It is difficult to accurately quantify the amount of DNA actually available for PCR amplification in a small sample. It was therefore essential to use different dilutions of normal control DNA (down to 1 × 10⁻⁴) and choose the dilution which produced a comparable yield, as estimated by agarose gel electrophoresis, to that of the tumour/serum/peritoneal fluid samples as the control lane for Genescan analysis.

LOH and MI
The use of Genescan software and multiple PCR runs allowed us to accurately measure alterations in allele ratios. LOH was defined as a >20% shift in allele ratios (see below), as calculated using the equation T₁:T₂/N₁:N₂ (where T₁:T₂ is the ratio of the two tumour alleles and N₁:N₂ is the ratio of the corresponding normal alleles). Novel alleles were defined as one or more novel peaks consistent in size with expansion or contraction of the microsatellite repeat and distinct from PCR stutter products. All LOHs or MIs were confirmed by multiple independent PCR amplifications.

RESULTS
Defining parameters for LOH
An important consideration in studies which look for LOH in tumour DNA is deciding what constitutes a significant shift in allele ratio at heterozygous loci. The use of Genescan Analysis software allowed us to accurately compare allele ratios from tumour, peritoneal fluid and serum DNA samples, with those obtained from normal DNA. Analysis of over 250 of these comparisons revealed a clear bimodal distribution, with 99% of values falling between 0 and 80% or 85 and 100% (data not shown), with a striking gap between 80 and 85%. On this basis we assumed that an allele ratio shift of 0–15% represented normal variation in the amplification of alleles present in equimolar amounts in normal diploid DNA, whereas a shift of >20% represented a notable deviation from a balanced allele ratio. For the purposes of this study we therefore chose a >20% shift as the criteria for defining LOH in our samples. However, most allelo-typing studies using microdissected paraffin-embedded tumour material tend to use a >50% shift to define LOH. We therefore also

Figure 1  Electropherograms of microsatellite instability (MI) or loss of heterozygosity (LOH) in three ovarian cancer patients. Fluorescently labelled PCR products were run on an ABI 373A DNA Sequencer. The fluorescent signals were converted into electropherograms by Genescan Software. Length of product in base pairs is indicated on the X-axis and an arbitrary measurement of peak height, proportional to signal intensity, on the Y-axis. (A) MI at p53V locus seen in patient 4224; (B) LOH at the D7S486 locus seen in patient 165; (C) LOH at the D5S346 (DP1) locus seen in patient 4571. N, normal DNA; T, tumour DNA; S, serum DNA; P, peritoneal fluid DNA.
analysed our results using a >50% shift in allele ratio as the criteria for LOH (see below).

**LOH and MI in primary tumour**

All of the primary tumours displayed at least one genetic alteration (Figure 1A–C), with the exception of patients 577, 0776 and 3944 (Table 1). LOH was detected at 39/80 (49%) informative markers, and was twice as common in stage II–IV (33/58, 57%) as in stage Ia tumours (6/22, 27%). LOH occurred most frequently at BRCA1 on chromosome 17q (63%), DP1 on 5q (56%) and D11 on 11p (53%) (Table 2).

Novel alleles were observed in the primary tumours of 3/6 stage Ia patients (3775, 4244, 065), but only in 1/14 patients (042) with more advanced disease (Tables 1 and 2). In the case of patients 3775 (novel alleles at 5/6 loci) and 4224 (2/6) this may be evidence of an RER+ phenotype associated with mismatch repair deficiency.

**LOH and MI in serum DNA**

Genetic alterations were detected in 17/20 serum DNA samples, including 5/6 of the samples from patients with stage Ia disease (Table 1). LOH was harder to detect in serum (27/83 or 33% of markers) than in the primary tumours (49%). Of the 27 cases of LOH detected in serum, 19 (73%) were observed in the corresponding primary tumour (Figure 1B,C) and eight were specific to the serum DNA (Table 2). Thus 19/39 (49%) examples of LOH found in primary tumours were detectable in serum DNA.

Novel alleles were harder to detect in serum DNA than in primary tumour DNA (Table 1). Of the nine examples found in primary tumours, four were detected in the corresponding serum samples (Table 2 and Figure 1A). The three patients with novel alleles in their serum DNA all had stage Ia disease (Table 1).

**LOH and MI in peritoneal fluid DNA**

Only 12 of the 19 DNA samples extracted from peritoneal fluid exhibited genetic alterations, and only 18/78 (23%) heterozygous markers displayed LOH compared to 33% in the serum samples and 49% in the primary tumours (Table 1). However, 17 of the 18 examples of LOH in the peritoneal fluid samples were also found in the corresponding primary tumour (Figure 1B,C), indicating a higher degree of clonality than was seen with the serum samples (Table 2).

Novel alleles were detected in peritoneal fluid DNA from two of the stage Ia patients, all of which had been observed in the primary tumour and serum from the same patients (Table 2 and Figure 1A). By definition, these two patients had negative peritoneal fluid cytology.

**Reanalysis of results using >50% in allele ratios**

Out of 84 examples of LOH featured in Table 1, 60 displayed >50% shift in allele ratio. Of the remaining 24, which showed a 20–50% shift, 19 coincided with the finding of a >50% shift in another sample from the same patient. For example, the serum sample from patient 4571 showed a 30% reduction in the shorter allele at marker DP1 (Figure 1C), whereas the tumour and peritoneal fluid samples showed almost complete loss. These observations of significant (P < 0.001) clonal similarity support our initial

### Table 2 Results of Genescan analysis of PCR products from 20 ovarian cancer patients analysed for LOH or microsatellite instability at six microsatellite loci

| Patient | Sample | DP1 | 522 | 486 | D11 | P53 | BRCA1 |
|---------|--------|-----|-----|-----|-----|-----|-------|
| 112     | Tumour | ○   | ○   | ○   | ●   | ○   | ○     |
|         | Serum  | ○   | ○   | ○   | ●   | ○   | ○     |
|         | Per. fluid | ○   | ○   | ○   | ●   | ○   | ○     |
| 200     | Tumour | ○   | ○   | ○   | ○   | ●   | ○     |
|         | Serum  | ○   | ○   | ○   | ●   | ○   | ○     |
|         | Per. fluid | ○   | ○   | ○   | ●   | ○   | ○     |
| 3775    | Tumour MI | MI | MI | MI | MI | ○ | ○ |
|         | Serum  | ○   | ○   | MI | x   | ○   | ○ |
|         | Per. fluid | ○   | ○   | MI | x   | ○   | ○ |
| 4224    | Tumour | ○ | x | ○ | MI | MI | ○ |
|         | Serum  | ○ | x | ○ | MI | MI | ○ |
|         | Per. fluid | ○ | x | ○ | MI | MI | ○ |
| 5475    | Tumour | ● | x | ○ | ● | ● | ○ |
|         | Serum  | ● | x | ○ | ● | ● | ○ |
|         | Per. fluid | ● | x | ○ | ● | ● | ○ |
| 065*    | Tumour | ● | MI | x | ○ | ○ | ○ |
|         | Serum  | ● | MI | x | ○ | ○ | ○ |
| 705     | Tumour | ○ | ○ | ○ | x | ● | ○ |
|         | Serum  | ○ | ○ | ○ | x | ● | ○ |
|         | Per. fluid | ○ | ○ | ○ | x | ● | ○ |
| 488     | Tumour | x | x | x | ○ | ● | ● |
|         | Serum  | x | x | x | ○ | ● | ● |
|         | Per. fluid | x | x | x | ○ | ● | ● |
| 165     | Tumour | x | ● | ● | x | ● | ● |
|         | Serum  | x | ● | ● | x | ● | ● |
|         | Per. fluid | x | ● | ● | x | ● | ● |
| 231     | Tumour | ● | x | ○ | ● | ● | ● |
|         | Serum  | ● | x | ○ | ● | ● | ● |
|         | Per. fluid | ● | x | ○ | ● | ● | ● |
| 042     | Tumour | ● | ● | ● | ● | MI | ● |
|         | Serum  | ● | ● | ● | ● | MI | ● |
|         | Per. fluid | ● | ● | ● | ● | MI | ● |
| 285     | Tumour | x | ● | ● | x | ● | ● |
|         | Serum  | x | ● | ● | x | ● | ● |
|         | Per. fluid | x | ● | ● | x | ● | ● |
| 577     | Tumour | ○ | ○ | ○ | ○ | x | x |
|         | Serum  | ○ | ○ | ○ | ○ | x | x |
|         | Per. fluid | ○ | ○ | ○ | ○ | x | x |
| 0776    | Tumour | x | x | x | ○ | ○ | ○ |
|         | Serum  | x | x | x | ○ | ○ | ○ |
|         | Per. fluid | x | x | x | ○ | ○ | ○ |
| 9930    | Tumour | ● | ● | ● | ● | x | x |
|         | Serum  | ● | ● | ● | ● | x | x |
|         | Per. fluid | ● | ● | ● | ● | x | x |
| 4992    | Tumour | ● | x | x | ○ | ○ | ○ |
|         | Serum  | ● | x | x | ○ | ○ | ○ |
|         | Per. fluid | ● | x | x | ○ | ○ | ○ |
| 5042    | Tumour | ● | ○ | ○ | ○ | x | ○ |
|         | Serum  | ● | ○ | ○ | ○ | x | ○ |
|         | Per. fluid | ● | ○ | ○ | ○ | x | ○ |
| 3944    | Tumour | ○ | ○ | ○ | ○ | x | ○ |
|         | Serum  | ○ | ○ | ○ | ○ | x | ○ |
|         | Per. fluid | ○ | ○ | ○ | ○ | x | ○ |
| 4571    | Tumour | ● | ● | ● | ● | x | x |
|         | Serum  | ● | ● | ● | ● | x | x |
|         | Per. fluid | ● | ● | ● | ● | x | x |
| 421     | Tumour | ● | ○ | ● | ● | ● | ● |
|         | Serum  | ● | ○ | ● | ● | ● | ● |
|         | Per. fluid | ● | ○ | ● | ● | ● | ● |

○ – heterozygosity retained; ● – loss of heterozygosity; MI – microsatellite instability; x – non-informative; NP – no product was obtained from repeated PCR amplifications. *No peritoneal fluid sample was available for molecular analysis from this patient. Per. fluid, peritoneal fluid.
assumption that a >20% shift represents a genuine case of LOH. However, taking a >50% shift as the criteria for LOH we would still have detected tumour-specific alterations in 5/6 serum samples from stage Ia patients but only 7/14 stage II–IV patients. In the case of peritoneal fluid we would still have detected alterations in 2/5 stage Ia samples, and 8/14 stage II–IV samples (reduced from 10/14).

LOH and MI in control cases

DNA was extracted from serum, peritoneal fluid and paraffin-embedded cyst wall samples from four patients with benign mucinous cystadenoma of the ovary and four with physiological ovarian corpus luteal cysts, as controls to evaluate the level of false positive results using this approach. The three samples from each of eight patients were analysed for alterations at all six loci by comparison with normal DNA extracted from white blood cells. Out of a total matrix of 144 genetic analyses, only two displayed genetic abnormalities; an MI at p53 and an LOH at 522, in the serum of a patient with a physiological cyst (results not shown).

DISCUSSION

We were able to detect genetic alterations in the free circulating DNA extracted from the serum of 17/20 (85%) patients with ovarian carcinoma, using a panel of six microsatellite loci. In addition, exfoliated cells from 12/19 (63%) peritoneal fluid samples were found to contain tumour-specific alterations. For such a limited panel of genetic markers this represents a relatively high degree of sensitivity. Given that all tumour cell populations will contain genetic alterations of some description it should be possible to approach 100% sensitivity using an optimized panel of genetic markers.

Significantly, it was possible to detect tumour-specific genetic markers in serum samples from five out of six patients with stage Ia disease. This suggests that detectable amounts of DNA are released into the blood from tumours confined to one ovary. Previous studies have shown that tumour DNA can be detected in the serum of patients with advanced head and neck (Nawroz et al, 1996), lung (Chen et al, 1996) and colon cancer (Hibi et al, 1998). This suggests that stage Ia ovarian tumours are more likely to have an RER+ phenotype than more advanced tumours. Studies on colorectal carcinoma show that patients with RER+ tumours have a better prognosis, because of reduced survival for cells with MI (Lothe et al, 1993; Lukish et al, 1998). In an analogous way the presence of DNA carrying novel alleles in serum may indicate higher levels of tumour cell death and signal a better prognosis for stage Ia ovarian cancer patients.

Two patients with stage Ia disease (3775 and 4224) could technically be upstaged to stage Ic as a result of tumour DNA being detected in their peritoneal fluid. These patients had been diagnosed as stage Ia on the basis of a negative peritoneal fluid cytology result and confinement of disease to one ovary histopathologically. Accurate staging has a significant bearing not only on prognosis, but also on the possible use of adjuvant chemotherapy in this patient subgroup. In the case of head and neck cancer, a prospective multicentre trial is currently underway in the USA to evaluate the efficacy of molecular staging and its impact on surgical practice (Caldas, 1997). To our knowledge, this is the first report of the possible molecular staging of ovarian cancer.

At present, population-based screening for epithelial ovarian cancer is not feasible due to the relatively low prevalence (15/100 000), the lack of specificity of available screening techniques, the absence of a definitive precursor lesion, and the inability to determine a lag time for the development of widespread disease. Advances in transvaginal sonography and colour Doppler imaging
(van Nagell et al., 1995), as well as the use of serial CA125 measurements (Skates et al., 1995) and complimentary serum markers such as O VX1 (Woolas et al., 1993), have improved early tumour detection. However, the efficacy of periodic screening of even high-risk patients has not been clearly established (Droegemueller, 1994). As molecular detection of tumour DNA in serum or peritoneal fluid seems possible even in stage Ia disease, this technique may hold considerable promise as a secondary screening tool after initial tumour marker or ultrasound abnormalities have suggested ovarian pathology. In terms of disease monitoring it will be interesting to observe if and when tumour DNA in the serum of these patients disappears after treatment, and to what extent its reappearance in serum predates clinical and biochemical evidence of recurrence.

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