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Original Paper

p53 Expression, DNA Content and Cell Proliferation in Primary and Synchronous Metastatic Lesions from Ovarian Surface Epithelial–Stromal Tumours

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The aim of this study was to investigate biological heterogeneity between primary and metastatic ovarian cancer lesions from individual patients as a means of elucidating steps in clinical progression. Cancer tissue from 61 untreated patients with ovarian surface epithelial–stromal tumours was examined. p53 expression detected immunocytochemically by the PAb1801 antibody, DNA content evaluated by flow cytometry, and cell proliferation evaluated as the [^{3}H]thymidine labelling index were investigated in primary tumours and corresponding synchronous metastases. The frequency of p53 positivity was similar in primary (62%) and metastatic (66%) sites, with an agreement between the two lesions from the same patient in 97% of the cases. Similarly, aneuploidy frequency (80%) and DNA indices were superimposable in primary and metastatic lesions from the same patient, with a 94% agreement. The frequency of aneuploidy was higher in p53-positive than in p53-negative lesions. An overall poor agreement ($r_s = 0.44$) was observed for proliferative activity of primary and metastatic lesions, due to a heterogeneous profile in omental with respect to primary tumours, which was mainly evident in p53-positive cancers. Conversely, cell proliferation of peritoneal, abdominal and pelvic lesions was qualitatively similar to that of the primary tumour in 88% of patients.

Key words: interlesional heterogeneity, ovarian surface epithelial–stromal tumour, ploidy, proliferative activity, P53, synchronous lesions

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INTRODUCTION

DIFFERENT PROFILES in genomic alterations, physiological and biochemical properties, growth and metastatic potential have been reported among different tumour lesions from the same patient [1–6]. Such biological interlesion heterogeneity is markedly evident in solid tumours and can be investigated to identify biological factors differently expressed by primary and metastatic lesions. It can consequently be used to detect preferential growth at the metastatic site of cell clones scarcely present in the primary tumour and to reconstruct the steps of tumour progression and metastatic spread [7–9].

In human ovarian surface epithelial–stromal cancers, the molecular mechanisms responsible for tumorigenesis and tumour progression are not well defined, although involvement of growth factors, proto-oncogenes, loss of heterozygosity on the 17p chromosome, and *TP53* gene alterations have been described [10–17]. Moreover, little information is available on interlesional heterogeneity of structural and/or functional alterations of oncogenes and tumour-suppressor genes within the same patient. Such information is important in order to determine whether gene alterations occur before metastatic spread or arise in the metastatic lesions, i.e. whether such events indicate a relatively stable phenotype or are necessary to support proliferation and growth at the metastatic site.

Alterations of the *TP53* gene have been observed in 30–70% of primary ovarian cancers [12, 14–16], whereas results on accumulation of the p53 protein are more homogeneous, from 50 to 70% [16–20]. The correlation between the immunohistochemical detection of p53 protein and the presence of

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mutations in the *TP53* gene has been analysed in some human tumour types including ovarian carcinomas [16, 18, 21–24]. Concordant results from the two assays have been observed in 86% of the cases [16], although some studies have shown that *p53* inactivation rather than gene mutation results in p53 accumulation [25, 26]. Overall, these data indicate the possibility of using immunohistochemistry as the least time-consuming and simplest approach for detecting *p53* alterations in ovarian surface epithelial–stromal tumour.

In the present study, p53 protein accumulation was analysed in 61 primary ovarian surface epithelial–stromal tumours and their synchronous metastases from untreated patients. In addition to p53 accumulation, other biological variables known to be indicators of prognosis and treatment response, such as DNA ploidy and cell proliferation [5, 27–29], were determined and analysed in parallel.

MATERIALS AND METHODS

Tumour material

Interlesional heterogeneity was investigated on primary ovarian surface epithelial–stromal tumours of various histological types and synchronous metastases from 61 untreated patients. 43 patients had tumours classified as stage III and 18 as stage IV, according to the FIGO classification. Most of the tumours (37, or 61%) were diagnosed as serous histotypes, 11 cases (18%) were endometrioid, and only 1 case was diagnosed as mucinous. Clear cells, undifferentiated or mixed histotypes were equally represented and accounted for the remaining 12 (20%) cases. Primary tumours and synchronous metastases were excised at the time of first-look laparotomy. Omental lesions were the most numerous (44 cases), followed by peritoneal, abdominal and pelvic lesions (6, 6 and 5 cases, respectively).

In vitro *determinations*

Immediately after surgery, part of the tumour material was incubated with [^{3}H]thymidine and then, after 6 h fixation in buffered-formalin, processed using conventional histological procedures for the determination of [^{3}H]thymidine labelling index (^{3}H-dT LI) and p53 expression. A part of the tumour material was frozen in liquid nitrogen and stored at −80°C for DNA ploidy determination.

^{3}H-dT labelling index. Samples were processed for the ^{3}H-dT LI determination as previously described by using a commercial kit (Ribbon, Milan, Italy) [27]. The ^{3}H-dT LI was evaluated independently by two observers by scoring a total of more than 3000 cells on consecutive areas from different specimens of the same tumour lesion, and was defined as the percentage ratio between labelled cells and total number of tumour cells. The median value of 7.5%, observed in previous unselected series [5] and which is similar to the median value observed in the present study for both synchronous lesions, was used as a cut-off to define slowly and rapidly proliferating tumours.

p53 immunohistochemical detection. Two histological sections of 4 μm from paraffin-embedded blocks were incubated for 1 h at room temperature with a 1:50 dilution of a murine monoclonal antibody, PAb1801 (Oncogene Science Inc., Manhasset, New York, U.S.A.) raised against human P53, which recognises both wild-type and mutant forms of P53 protein. After incubation, the specimens were processed by using the ABC immunoperoxidase system (Vectastain ABC Kit, Vector Laboratories, Burlingame, California, U.S.A.) as previously described [30]. A set of ovarian surface epithelial–stromal tumours with high levels of P53 were used as positive controls. Negative controls were obtained by omission of the primary antibody. Positivity was exclusively nuclear. Quantification was carried out microscopically by two independent observers, scoring a total of 1000–3000 tumour cells on consecutive areas from different specimens of the same tumour lesion. Positivity was expressed as the percentage ratio between stained cells and total number of tumour cells. To define p53-positive (p53$^+$) tumours, we used a cut-off of 5% of positive cells, which has already provided prognostic information in other human tumour types [30].

DNA content analysis. Samples were processed for DNA flow cytometric analysis as previously described [31] and analysed with a FACScan (Becton Dickinson, Sunnyvale, California, U.S.A.). For each tumour lesion, 30000 events were acquired. Human lymphocytes, used as an internal standard, showed a median coefficient of variation of 1.5% for the $G_{0/1}$ peak. Tumours with a DNA index (DI) lower than 0.95 or higher than 1.05 and with more than 10% aneuploid cells were considered aneuploid. If aneuploid, a tumour was considered tetraploid when the cells in the region corresponding to diploid G_2 and tetraploid $G_{0/1}$ accounted for more than 20% of the total number of analysed cells and when a peak corresponding to G_2 of a tetraploid population was present. When two or more aneuploid cell populations were present, the DI of the largest aneuploid population was chosen as representative of the tumour.

Statistical analysis

The relationship between the profiles of biological variables in the primary tumours and in their synchronous metastases was assessed by the Spearman rank correlation coefficient (r_s) on the overall series of cases and on subsets defined by the anatomical site of metastasis. The statistical significance of biological differences among the lesions was assessed by means of the Wilcoxon rank-sum test (^{3}H-dT LI) or by the chi-square test (p53 expression or ploidy status).

RESULTS

p53 expression, DNA ploidy and ^{3}H-dT LI in the primary tumour and its synchronous metastases

Biological information on the primary tumour and its synchronous metastasis was available in all 61 cases for P53 expression and ^{3}H-dT LI, but only in 50 cases for DNA ploidy.

Overall, immunostaining for p53 was similar in primary and metastases and, within the latter lesions, was independent of the metastatic site. Specifically, more than 5% of p53$^+$ cells were detected in 62% of primary tumours and in 66% of metastatic lesions, and 1% to 5% of p53$^+$ cells were found in 8% of primary tumours; 30% of ovarian cancers and 34% of metastases showed no reactivity. A high agreement in the expression of p53 was observed between primary and metastatic lesions from the same patient ($r_s = 0.87$, $P = 0.0001$; Figure 1), regardless of the site of the metastasis. However, in the subset of 38 tumours with both lesions showing p53 positivity (i.e. more than 5% of p53$^+$ cells), a trend of a higher frequency of p53$^+$ cells was observed in metastatic lesions than in primary tumours (median values, 32.6% versus 22.8%;

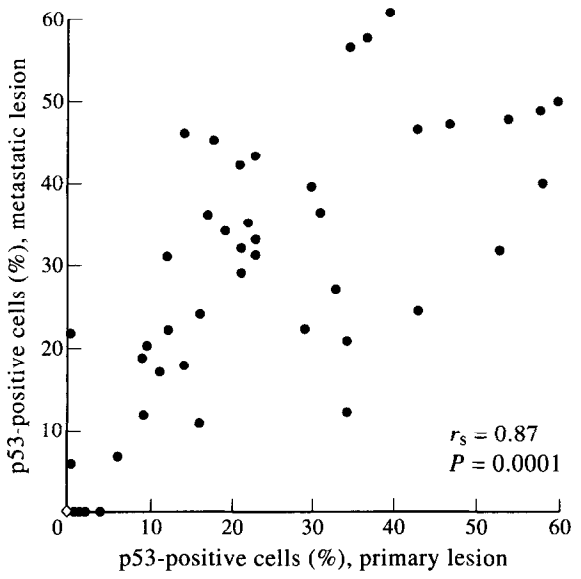

Figure 1. P53 expression in the primary tumour and its synchronous metastatic lesion (61 cases). 17 cases (empty rhombus) showed no immunoreactive cells in primary or metastatic lesions.

$P = 0.06$). A qualitative interlesional agreement in P53 positivity was observed in 97% of the cases. Discordance was observed in 2 cases, which exhibited $p53^+$ cells (6% and 22%) only in the metastatic lesions.

Aneuploid cell populations were detected with the same frequency on primary (82%) and metastatic (80%) lesions. Within metastatic lesions, the highest frequency of aneuploidy was observed in omental (89%) and the lowest frequency (1/5) in the few pelvic lesions ($P = 0.004$). A high agreement between primary and metastatic lesions from the same patient was observed in ploidy status (94%) and DI values ($r_s = 0.81$, $P = 0.0001$; Figure 2). Qualitative disagreements were due to the detection of aneuploidy only in the primary or only in the metastatic lesions in 2 and 1 case, respectively.

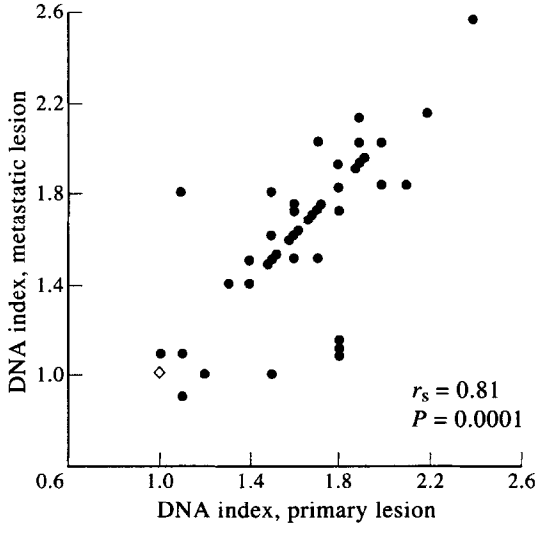

Figure 2. DNA index of a primary tumour and its synchronous metastatic lesion (50 cases). 8 cases (empty rhombus) showed primary and metastatic lesions with a DI greater than 0.95 or lower than 1.05.

Overall, the median ^{3}H-dT LI value was not significantly different in primary and metastatic lesions (8.2% versus 6.7%). Conversely, within metastatic lesions, the lowest ^{3}H-dT LIs were observed in omental compared to the other metastatic sites (median values, 6.3% versus 10.7%; $P = 0.03$). When ^{3}H-dT LI values of primary and metastatic lesions from individual patients were matched, a poor correlation was observed, with a marked dispersion of experimental data ($r_s = 0.44$). A breakdown analysis as a function of the site of metastasis showed a strong and significant direct correlation in proliferative activity for the subset of peritoneal, abdominal and pelvic lesions ($r_s = 0.74$, $P = 0.0006$; Figure 3a) and a poor agreement for omental lesions ($r_s = 0.29$; Figure 3b). Such a difference in proliferative profiles between the primary tumour and the different metastases was paralleled by a similar difference when lesions were simply classified as slowly or rapidly proliferating by using the median ^{3}H-dT LI as cut-off value. Based on such a classification, the qualitative agreement was minimal for omental lesions (50%) due to a lower cell proliferation in metastatic lesions than in the relative primary tumour (Table 1), and maximum for the other metastases (88%).

Relationship between p53 expression, DNA ploidy and ^{3}H-dT LI in primary and metastatic lesions

Absence or low levels of p53 expression were more frequently associated with euploidy and p53 accumulation to aneuploidy ($P = 0.01$). In particular, the association between p53 accumulation and DNA ploidy was observed in about two-thirds of the cases, regardless of lesion type (66% in the primary lesions and 72% in the metastases). Conversely, no relation was observed between ^{3}H-dT LI and p53 expression or DNA content, in primary or metastatic lesions. However, p53 accumulation seemed to be associated with proliferative interlesional heterogeneity (Table 1). In fact, in the overall series, the concordance between ^{3}H-dT LI of primary and metastatic lesions was higher in p53-negative ($p53^-$) tumours than in $p53^+$ tumours, considering ^{3}H-dT LI as a continuous ($r_s = 0.66$ and 0.32, respectively) or as a dichotomous variable (agreement rate: 74 and 53%, respectively).

The evidence of a proliferative heterogeneity between primary and metastatic lesions, as a function of metastatic site and p53 accumulation, induced us to analyse the proliferative modulation of a primary tumour to its synchronous metastasis as a function of both factors (Table 1). By considering ^{3}H-dT LI of the primary and metastatic lesions as a continuous variable, Spearman's correlation coefficients were similarly high for peritoneal, abdominal and pelvic lesions, regardless of p53 status of the primary tumour, and for omental lesions only for $p53^-$ tumours. Conversely, no relationship was observed between ^{3}H-dT LI of $p53^+$ tumours and their omental lesions in 17 of the 28 cases, with a proliferative increase from the primary to the metastasis in 7 cases and a decrease in the remaining 10 cases.

DISCUSSION

Studies on the biological heterogeneity between primary and metastatic lesions from individual patients should add to the knowledge of the successive steps of biological and clinical progression. Their speculative and applicative implications are markedly increased by available information on the molecular

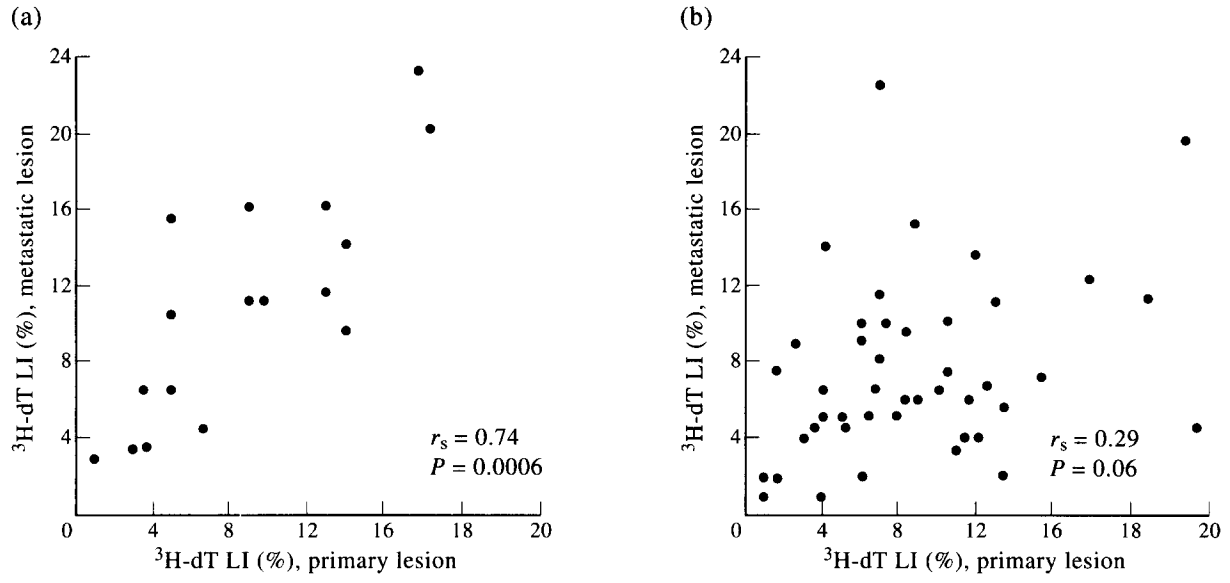

Figure 3. ^{3}H-dT LI of a primary tumour and its synchronous metastatic lesion in the same patient (61 cases). The analysis was separately performed in (a) peritoneal, pelvic and abdominal lesions (17 cases) and in (b) omental lesions (44 cases).

Table 1. Variations in proliferative activity between primary tumour to synchronous metastasis as a function of metastatic site and P53 expression

	Percentage of cases with different ^{3}H-dT LI profiles in primary/metastatic lesions						Correlation coefficient (r_s)
	Agreement			Discordance			
	Low/low	High/high	Total	Low/high	High/low	Total	
Metastatic site							
Omental (n = 44)	30 (13)†	20 (9)	50 (22)	18 (8)	32 (14)	50 (22)	0.29
Others* (n = 17)	35 (6)	53 (9)	88 (15)	12 (2)	0	12 (2)	0.74
P53 expression							
P53$^-$ (n = 23)	39 (9)	35 (8)	74 (17)	9 (2)	17 (4)	26 (6)	0.66
P53$^+$ (n = 38)	26 (10)	26 (10)	53 (20)	21 (8)	26 (10)	47 (18)	0.32
Omental lesions							
P53$^-$ (n = 16)	31 (5)	38 (6)	69 (11)	6 (1)	25 (4)	31 (5)	0.71
P53$^+$ (n = 28)	29 (8)	11 (3)	39 (11)	25 (7)	36 (10)	61 (17)	0.10
Other lesions*							
P53$^-$ (n = 7)	57 (4)	29 (2)	86 (6)	14 (1)	0	14 (1)	0.61
P53$^+$ (n = 10)	20 (2)	70 (7)	90 (9)	10 (1)	0	10 (1)	0.74

*Peritoneal, abdominal, pelvic. †Number of cases in parentheses.

mechanisms responsible for tumour development and progression [7–9]. However, most studies have been performed on experimental tumours, and objective problems make it difficult to obtain substantial information on the spatial and temporal biological profiles of clinical lesions from the same patient.

In a previous study on a small number of patients with ovarian stromal epithelial–stromal tumours [5], we observed that the consideration of cell kinetics of both primary and synchronous lesions can improve the prognostic accuracy compared with analysing the cell kinetics of the two lesions separately. In the present study, we again focused on cell proliferation, but phenotypic (DNA ploidy) and functional aspects (p53 expression) were also evaluated on a larger series of primary and synchronous metastatic lesions at diagnosis, before any clinical treatment.

Increasing evidence supports the involvement of p53 inactivation in induction and progression of ovarian surface epithelial–stromal tumours [14, 15, 17, 32], although the molecular mechanisms involved have not yet been clarified, in contrast to other tumours such as colorectal carcinoma [9]. p53 inactivation has been reported to occur before metastatic spread, and it has been suggested that it is largely maintained during progression [15]. On the whole, our data indicate that the level of p53 protein, as detected immunocytochemically by PAb1801 in primary ovarian cancer, was similar to that of the metastasis. Since, in ovarian cancer, p53 accumulation correlated, in a high percentage of cases, with the presence of

mutations [16], our findings showed that alterations in the *p53* gene indicate a relatively stable phenotype, and that disease dissemination is not usually associated with a gain of *p53* aberrations, in agreement with results published on other tumour types [33]. We observed an increase in the percentage of p53-accumulating cells in metastatic lesions compared to their primary tumours. Such a finding could be explained by a positive selection or by an advantage for $p53^+$ cells during the invasion process.

The strong correlation between p53 expression and ploidy status, observed in about two-thirds of the cases, is in keeping with results previously reported by other authors for ovarian carcinoma [16, 18]. Along with other experimental evidence [12, 34], it supports the hypothesis that p53 wild-type protein inactivation may be responsible for a genomic and chromosomal instability of tumour cells. As already reported for ovarian surface epithelial–stromal tumours and other tumour types [35, 36], ploidy status proved to be a stable feature between primary and metastatic lesions. Similar to that observed in other tumours and in different clinical situations (synchronous or metachronous lesions, with and without intercurrent treatments), cell proliferation appeared as a functional marker able to reflect clinical progression and treatment modulation at a biological level [4, 37, 38].

In our experience, proliferative profiles were markedly heterogeneous in primary and synchronous metastatic lesions, mainly for omental metastases. Within this latter subgroup, the heterogeneity was mostly evident in $p53^+$ cancers, possibly suggesting a high susceptibility of kinetically heterogeneous tumours to develop *p53* mutations and/or protein accumulation. However, although such a hypothesis is the most likely to explain our results, it remains to be assessed whether *p53* alterations actively contribute or are only correlated with the proliferative deregulation between the primary tumour and omental metastasis. Interestingly, even in ovarian surface epithelial–stromal tumours, as already observed in breast cancers [30], p53 expression appears to be independent of cell proliferation, thus supporting that the role of *p53* alteration is not restricted to the loss of cell cycle control [39].

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