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Immunochemical Analysis of the p53 Oncoprotein in Matched Primary and Metastatic Human Tumours

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There is much interest in the range of genetic aberrations which occur in human malignancies. An immunohistochemical study has been carried out to investigate the consistency of expression of abnormally accumulated p53 protein in paired samples of archival primary and metastatic carcinomas. The staining of methacarn-fixed tissue from 136 matched pairs of mammary carcinoma and 20 cancers from other sites was completed using antibody CM-1 and DO1 in a sensitive peroxidase-conjugated streptavidin-biotin technique. The majority of tumour cells were positive in 25% and the tumours were negative in 17% of the primary carcinomas; staining was heterogeneous in the remaining cases. Staining was identical in 180/186 (96%) metastatic lesions. An ELISA assay carried out on 12 matched pairs of the tumour specimens demonstrated that altered conformation of the aberrant p53 protein present in a primary lesion was maintained in its metastasis. These data indicate that alterations in the p53 gene result in a relatively stable phenotype and that progression of disease is not usually accompanied by either further mutation or loss of the mutant allele.
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INTRODUCTION

THERE IS compelling support for the notion that carcinogenesis represents a multistep process. Cancer statistics relating the tumour incidence with age indicated that some five or six steps or 'hits' are required for a common human tumour to reach a diagnosable stage [1]. More recently, the cytogenetic and molecular biology analyses have confirmed the long anticipated presence of a range of genetic aberrations in human malignancies [2, 3]. Among the most striking of such alterations are mutations in the p53 gene [4, 5]. At present, the p53 mutations are regarded as the most frequent genetic change so far identified in human cancer and the genetic and biological features of p53 show a puzzling combination of a tumour suppressor gene and a dominant oncogene [4, 5]. The p53 mutations are usually point missense mutations in the phylogenetically highly conserved central region of the molecule [5, 6]. They appear to be important both in the pathogenesis of a broad range of sporadic tumours [5, 7, 8], and as germline mutations representing the inherited basis

of the Li–Fraumeni cancer family syndrome [9]. Although it is clear from the latter example that p53 aberrations can serve as the earliest initiating event in the stepwise process leading to cancer, such mutations are often regarded as a relatively late step, contributing to progression in the development of common sporadic cancers like carcinomas of the colon [10]. Since it is the accumulation of several genetic changes rather than their precise order which eventually leads to malignant phenotype [2, 3], it is feasible to speculate that a p53 mutation can play an important role at any stage of tumorigenesis. In fact, the only stage of carcinogenesis to which the contribution of p53's aberrations has not been properly investigated and documented is the transition from a primary to a metastatic lesion.

Another striking feature of the p53 point mutations is a dramatic increase in the stability of the protein leading to high p53 levels in cancer cells [11–17]. This well-established association of point mutations with accumulation of p53 protein has important practical consequences in that simple immunohistochemical methods can provide strong evidence of mutations in the p53 gene. It must be recognised, however, that in addition to mutation, both alleles can be lost and in this case there is no protein production. Such indirect evidence for the presence of p53 mutations can be further supported by immunochemical examination of the p53 protein's conformation, since a common conformational change characterised by a selective exposure of the PAb240 epitope appears to be associated with many point mutations found in animal [18] and human [12, 14] cancers. The immunochemical and immunohistological analyses of the p53 oncoprotein have recently been significantly aided by the development of novel monoclonal and polyclonal anti-p53 antibodies which are effective even on paraffin-embedded tissue sections [8, 19–21].

In the present study, we have applied the new anti-p53 immunoreagents for both immunohistochemical and ELISA analyses of the p53 protein in a large series of primary human tumours and their corresponding metastases. The questions we sought to address by such investigation were two-fold: (1) is there any evidence for 'gain of p53 alterations' at the metastatic stage as compared with the primary tumour which could indicate that the p53 change can contribute to tumorigenesis at such a late stage as the transition to metastatic spread; (2) are the p53 protein aberrations (accumulation patterns, conformation) found in a primary tumour also faithfully preserved in one or more metastatic lesions derived from it? The answer to the later question could shed some light on the more basic problem of whether the aberrant p53 proteins merely represent functional inactivation or whether they also provide some positive oncogenic stimulus associated with a selective growth advantage, in which case such accumulated mutant proteins would be maintained rather than randomly lost in the late stages of the cancer progression.

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MATERIAL AND METHODS

Tissues

Samples of tissue were processed within 1–2 h after surgery. Samples of all specimens were fixed in methacarn (a mixture of methanol, chloroform and acetic acid, 6:3:1, by volume) for 2–12 h at room temperature and then processed and embedded in paraffin wax. Some specimens were also immediately flash-frozen in liquid nitrogen and kept at −80°C. The material used in the present study consisted of a series of 133 matched samples of primary and secondary infiltrating ductal and lobular breast carcinomas and a further 10 pairs of primary and recurrent breast tumours of varying histological types (see Table 1 for more details) and a small heterogeneous group of similar pairs of primary cancers and corresponding metastases from solid tumours from other sites including colorectal carcinomas (n = 6), melanomas (n = 5) and one pair of each of gastric and vulval carcinomas. In 30 of the above-mentioned cases (29 breast and one gastric carcinoma), samples from two or more independent metastatic deposits were available for this analysis resulting in a total number of 156 primary and 188 metastatic lesions examined. The spectrum of metastatic sites included axillary lymph nodes (n = 109), skin and subcutaneous tissues (n = 52), supraclavicular lymph nodes (n = 11), draining lymph nodes of other locations (n = 14) and solid pleural metastases (n = 2), in addition to the 7 cases of recurrent breast carcinoma.

Antibodies

The anti-p53 mouse monoclonal antibodies PAb240 which recognise a conformation-dependent epitope specifically exposed in the majority of p53 mutant proteins and Bp53-11 which recognises an epitope near the N-terminus accessible in both wild type and mutant p53 proteins, have both been

Table 1. p53 immunostaining patterns in primary tumours

Tumour type	Total no. examined	Immunostaining patterns† Negative	Rare +ve	Hetero-geneous +ve	Homo-geneous +ve
Breast carcinomas					
Invasive ductal	123	20	43	28	32
Invasive lobular	10	4	3	3	0
Other malignant breast tumours*	10	1	4	3	2
Colon carcinomas	6	0	2	0	4
Melanomas	5	1	2	2	0
Gastric carcinoma	1	0	0	0	1
Vulval carcinoma	1	1	0	0	0
Total tumours	156	27 (17%)	54 (35%)	36 (23%)	39 (25%)

*This group of breast tumours included medullary carcinomas (n = 3), tubular carcinoma (n = 1), adenoid cystic carcinomas (n = 2), apocrine carcinomas (n = 2), carcinoma with squamous metaplasia (n = 1) and cystosarcoma phyllodes (n = 1).
†Negative = no obvious positive cells; rare positive = up to 5% positive tumour cells; heterogeneously positive = between 5 and 70% positive tumour cells; homogeneously positive = more than 70% positive tumour cells.
The results obtained with each of the anti-p53 antibodies are not listed separately in the table since they were essentially identical. All cases were stained with CM-1 and DO-1. Identical results were obtained with each antibody.

described previously [18, 19]. The CM-1 is a rabbit high titre polyclonal antiserum recently raised against human recombinant wild type p53 protein [20] and the DO-1 is a novel mouse monoclonal antibody recognising an epitope near the *N*-terminus of the human p53 protein [21]. While the PAb240 only works satisfactorily on frozen tissue sections, the antibodies Bp53-11, DO-1 and CM-1 also give very good immunostaining results on paraffin-embedded material. The control reagents included monoclonal antibodies to keratins and vimentin (positive control) and rabbit and mouse pre-immune sera or an irrelevant IgG mouse monoclonal antibody (used as negative controls).

Immunohistochemistry on paraffin sections

The sections were floated onto poly-L-lysine-coated slides and allowed to dry overnight at room temperature [20]. The dewaxed sections were rehydrated in several changes of phosphate buffered saline (PBS), non-specified binding was blocked with 10% serum (the origin depending on the primary antibody and detection kit used) and primary antibodies applied and incubated overnight at 4°C. We routinely used the CM-1 antiserum diluted 1:1500 and the monoclonal antibodies as hybridoma tissue culture supernatants at 1:10 dilution. Vectastain Elite detection kit based on the sensitive biotin-streptavidin-peroxidase system was employed as recommended by the manufacturer, with 3,3'-diaminobenzidine in 0.03% nickel sulphate as chromogen. In the present study, all cases were stained with DO-1 and CM-1 antibodies and about 60% of the sections also with BP53-11.

Plate immunoassays

Frozen tumour tissues were pulverised in a mortar and the powder was then lysed in 150 mmol/l NaCl, 50 mmol Tris (pH 8.0), 5 mmol/l EDTA, 1% NP40, 1 mmol/l phenylmethylsulphonyl fluoride for 30 min on ice, followed by centrifugation at 15 000 *g* for 30 min in the cold to clear the extract. Falcon 96-well microtitre plates were incubated overnight with 50 μl per well of 20 μg/ml of purified capture antibody (PAb240 to detect the altered 'mutant conformation' and DO-1 to detect total p53 levels), rinsed in PBS and blocked for 3 h in 3% bovine serum albumin (BSA) in PBS. The blocking solution was then removed, 50 μl of tissue extract prepared as above, and serial dilutions in lysis buffer were added to each well and incubated for 2 h at 4°C. The plates were washed three times in 0.1% NP40 in PBS and once in PBS, and polyclonal antiserum CM-1 diluted 1:1500 was added for additional 2 h. The plates were washed as above, incubated for 2 h with peroxidase-conjugated swine antiserum to rabbit immunoglobulin (Dako, diluted 1:1000), the peroxidase reaction visualised with tetramethylbenzidine, the reaction stopped by adding 50 μl per well of 1 N H_2SO_4 and the results recorded in a plate reader at 450 nm. The extracts from cell lines known to contain high levels of p53 of known conformations [12] were used in control assays, negative control immunoreagents included irrelevant monoclonal antibodies (to replace the solid phase capture antibodies) and pre-immune rabbit serum (to replace CM-1). All assays were performed in duplicate. The quantitative relationship between optical density units and the estimated amount of p53 protein present in the ELISA assay has been established by our group and the relationship is linear over the range of protein concentrations used in the assay [22].

RESULTS

Patterns of p53 expression in primary tumours

The histological classification of the 156 primary tumours examined, the numbers of cases in each group and the immunostaining patterns obtained with the anti-53 antibodies are summarised in Table 1. Similar staining patterns were observed with both antibodies. Based on our previous experience with p53 immunohistochemistry on a variety of primary solid tumours [8, 13, 19, 20] as well as on the results obtained in this study, we have divided the staining patterns observed into four basic categories. The most striking pattern was an intense nuclear granular staining confined to cancerous cells and present in the majority (> 70%) of the tumour cells. This homogeneous pattern was seen in about 25% of the primary tumours and a representative example of such staining is shown in Fig. 1a. In 23% of primary neoplasms, the p53 immunostaining was considerably more variable, with respect to both the staining intensity and percentage of positive tumour nuclei (heterogeneous pattern). In a significant proportion of the sections examined (35%), only a small fraction (5% or less) of tumour nuclei was positively stained. This category also included some tumours with rare strongly positive nuclei scattered throughout the neoplasm (Fig. 1c). The fourth pattern, namely the complete absence of reaction, was found in 17% of primary cancers (Table 1). All three patterns characterised by at least a fraction of p53-overexpressing nuclei were specific to the tumour area and, therefore, represent some tumour-specific aberration. The subclassification into several categories (staining patterns) permits more precise comparison of the primary lesions with their corresponding metastases.

Finally, since this study was primarily aimed at evaluation of p53 changes at distinct stages of tumour progression, we also examined the p53 staining patterns in the *in situ* vs. infiltrating component which were present together on the same section in 69 of the 143 primary breast carcinomas. Of these 69 carcinomas, 60 showed at least a fraction of tumour nuclei with detectable accumulation of p53 in their infiltrating component. When the carcinoma *in situ* and infiltrating carcinoma areas within each lesion were compared, 56 cases (93%) showed similar p53 staining patterns in both components of the tumour and only 4 cases (7%) were found to be positive in their invasive component while negative in the *in situ* carcinoma areas. In no case was a reverse 'discrepancy' observed (p53-positive *in situ* component but negative adjacent infiltrating tumour).

p53 staining patterns are maintained in metastatic lesions

The comparison of p53 expression in 188 metastatic lesions with the staining patterns found in the corresponding primary tumours revealed very good correlation between the two sets of data (Table 2). In particular, there were no 'gain-of-positivity' metastases found in the whole series. Furthermore, in only 1 case was there an increase in staining: one lymph node metastasis showed the heterogeneous pattern as opposed to the only rarely p53-positive nuclei in the matched primary ductal carcinoma of the breast. The vast majority of the cases showed the same p53 immunostaining pattern in the primary neoplasm and in one or more secondary lesions derived from it (Fig. 1b, d; Table 2). Total concordance with regard to p53 expression was also found between the seven recurrent breast carcinomas and the original lesions. As to the potential losses of p53 accumulation during the transition from primary to secondary tumour, there were four local (axillary lymph nodes) and two distant metastases which showed no detectable staining while there were some rare p53-positive nuclei in the matched primary lesions (Table 2). The only case of a more dramatic loss (a change from the heterogeneous to negative phenotype) was detected in one of the two distant metastases derived from a medullary breast

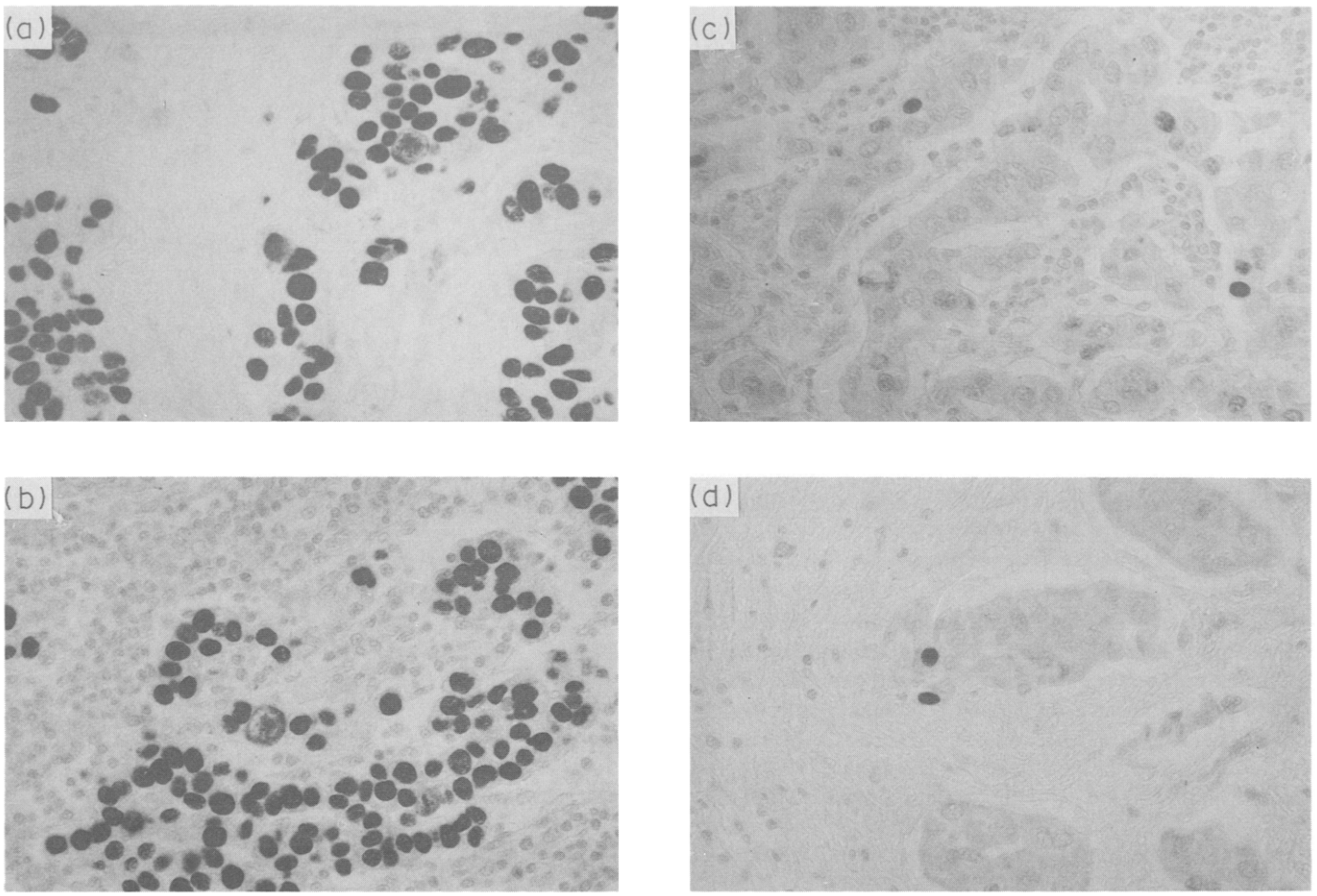

Fig. 1. Immunohistochemical detection of p53 in breast carcinomas. Strong homogeneous staining pattern in a primary (a) and corresponding metastatic lymph node lesion (b). Rare scattered positive nuclei in a primary carcinoma (c) and its subcutaneous metastasis (d). Staining with DO-1 (a, b) and CM-1 (c, d). Magnification × 400.

carcinoma. It is worth mentioning that in all cases where the primary tumour showed the most positive homogeneous pattern, the corresponding metastases scored also in this category. In general, over 95% of the 188 metastases examined in the present study faithfully preserved the p53 protein alterations detected in the matched primary cancer. In addition, despite several cases of a moderate loss of p53 staining among metastases, there was no significant difference in the frequency of such events in local (4%) as compared with distant (3%) secondary lesions (Table 2).

Conformational alteration of p53 is preserved in metastases

Mutation of the p53 gene is often accompanied by conformational alteration of the protein, characterised by the selective exposure of the PAb240 epitope [8, 12, 14, 18]. The proportion of aberrant p53 protein with such altered conformation can vary widely and appears to be a characteristic feature of each precise mutation. To further support and extend the immunohistochemical data described above we sought to employ the sensitive ELISA technique to investigate whether the staining patterns correspond to elevated p53 protein levels in matched tumour

Table 2. p53 immunostaining patterns in metastatic lesions as compared with the matched primary tumours

Type of lesion	Number examined	p53 staining pattern related to primary tumour		
		Identical	Gain of positivity*	Loss of positivity†
Local metastases	123	118	1	4
Distant metastases	65	62	0	3
Recurrent tumour	7	7	0	0
Total no. (%)	195	187 (96%)	1 (0.5%)	7 (3.5%)

*Gain of positivity was seen as a transition from a rare positive pattern in the primary carcinoma to a heterogeneous pattern in the matched lymph node metastasis.
†Loss of positivity cases include seven negative metastases of which the corresponding primary lesion was classified as rare positive ($n = 6$) or heterogeneous ($n = 1$).

homogenates and, in particular, whether the altered conformation of p53 is maintained during progression to metastatic spread. Due to the amount of tissue required for this analysis it was limited to a total of 31 tumour specimens. In 7 cases either primary or secondary (but not both) lesions were available (four breast carcinomas, two colon carcinomas and one melanoma) and these samples were merely used to establish relative p53 protein levels in tumour extracts for correlation with immunohistochemistry. The remaining 24 samples represented 12 pairs of matched primary and secondary tumours (eight breast carcinomas, two colon carcinomas, one gastric carcinoma and one melanoma, see Table 3). The later series was used for both the estimation of p53 levels and evaluation of p53 conformation. Of the 25 extracts analysed the ELISA method was able to detect clearly elevated p53 protein in all 11 samples classified as homogeneously positive and also in the seven specimens scored as heterogeneous by immunostaining (Fig. 2a).

The p53 protein levels measured in the ELISA assay were indistinguishable from background levels in the four immunohistochemically negative samples and in the six specimens with only a small fraction (about 3% or less) of positive nuclei (Fig. 2a). Of the 12 pairs of matched primary and secondary cancers, three pairs exhibited low levels of staining and were negative by ELISA, whilst nine showed clear p53 accumulation and were, therefore, amenable to conformational analysis (Table 3). The tumour extracts were examined in a plate immunoassay based on monoclonal antibodies recognising total (DO-1) or altered (PAb240) p53 protein. The fraction of p53 in the '240 conformation' varied from case to case but did not differ significantly from the given primary cancer and its metastasis (see Fig. 2b, c). The overall good agreement between the proportion of the conformationally altered p53 within the matched specimens is more obvious in the summary graph shown in Fig. 3. Taken together the immunochemical analysis of p53 in tumour extracts demonstrated good correlation of p53 protein levels with the immunohistochemically defined staining patterns and showed that the altered conformation characteristics of aberrant p53 found in primary cancers is preserved in the corresponding metastatic lesions.

DISCUSSION

Metastasis is the most dangerous phase of the multistep process of carcinogenesis. Although a range of oncogenes can confer the ability to metastasise together with some other features

Table 3. Correlation between immunohistochemical staining for p53 and immunochemical demonstration of p53 by ELISA assay

	ELISA +ve/ homogeneous + heterogeneous staining	ELISA −ve/ low level + negative staining
7 cases either 1° or 2°		
4 breast		
2 colon	3	4
1 melanoma		
12 paired samples of 1° and 2°		
8 breast	6	2
2 colon	2	—
1 gastric	1	—
1 melanoma	—	1

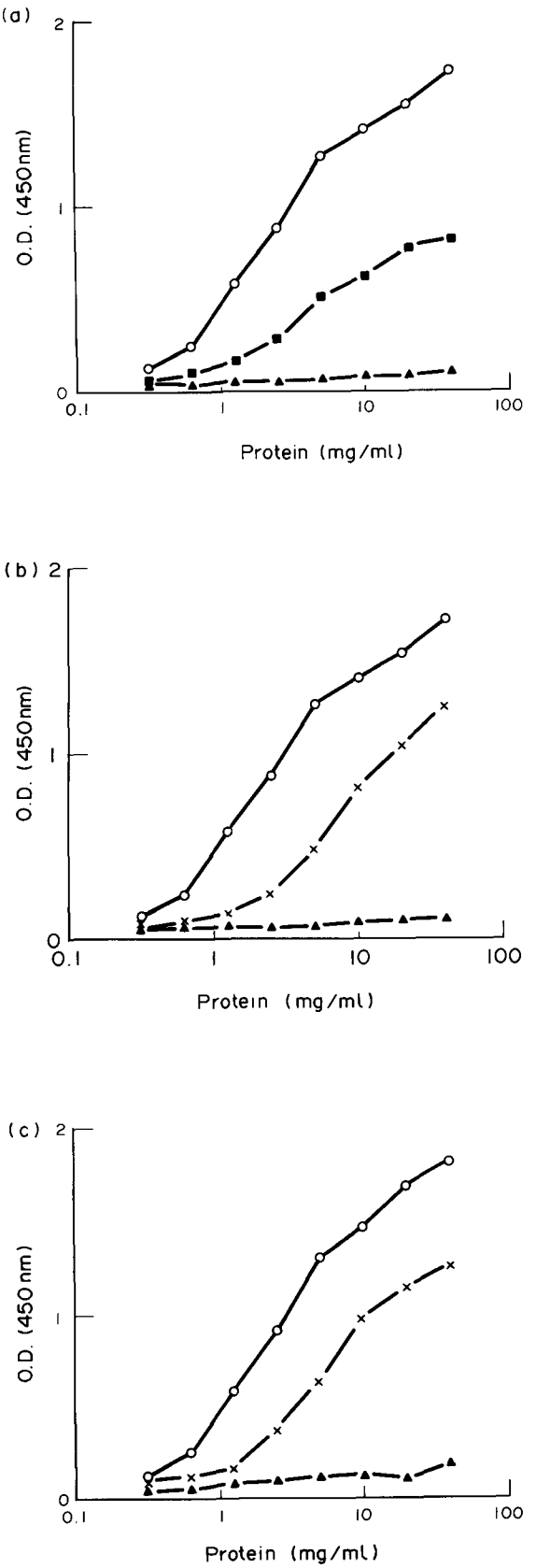

Fig. 2. Sandwich ELISA data demonstrating levels and conformation of p53 in tumour extracts. (a) Accumulation of p53 is detectable in homogeneously (○–○) and heterogeneously (■–■) immunostaining tumours but undetectable in a carcinoma with scattered (~ 1–2%) positive nuclei (▲–▲). (b, c) Total levels (○–○) and '240 (mutant)' conformation fractions (X–X) of p53 in a primary (b) and matched secondary (c) carcinoma. (▲–▲) = negative control.

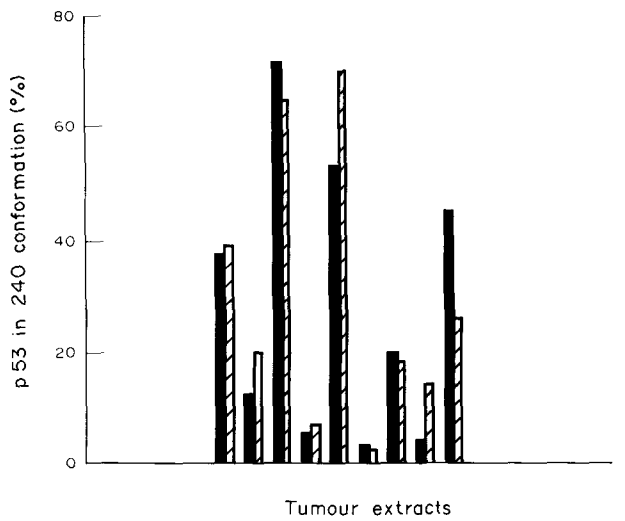

Fig. 3. Summary graph showing the general tendency to preserve proportions of the '240 conformation' in corresponding pairs of primary (■) and secondary (▨) tumours. The data are expressed as percentage of the '240 conformation' in total p53 levels at the tumour extract protein concentration 40 mg/ml.

of malignancy [23], there has been little progress in attempts to identify the genes that can elicit metastases as their only contribution to carcinogenesis. To date, perhaps the most likely candidate gene associated with metastasis is NM23, the loss of which appears to play an important role in the secondary spread of human tumours [24]. The question we addressed in this study was whether aberrations of p53, the gene frequently altered in a variety of common human cancers, can occur as late as the onset of metastasis. The available evidence clearly shows that the p53 mutation can be an initiating or early event in carcinogenesis [9, 15, 19, 25] and can also occur later, during progression of low-grade to higher grade tumours [2, 10, 26, 27]. In contrast, the potential role of p53 alterations in the process of metastasis has not been explored in detail though p53 expression in small series of primary and corresponding secondary neoplasms has been compared in breast [15], stomach [28] and lung [29] tumours. Our present data based on the p53 protein expression patterns and conformation suggest that the genetic changes leading to altered p53 expression occur well before the onset of metastasis and do not provide any evidence to support a role for p53 in metastatic spread. This conclusion is also in agreement with limited DNA sequencing data available for a few matched pairs of primary and secondary breast carcinomas [15] and small-cell carcinomas of the lung [29]. In addition, the almost invariable preservation of the altered p53 characteristics in both local and distant metastases when compared with the relevant primary lesions found in the present study is consistent with the hypothesis that the p53 alterations are essential for the maintenance of the malignant phenotype in the progression of human cancer including the process of metastasis.

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