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Angiogenesis in Primary Lung Cancer and Lung Secondaries

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INTRODUCTION

THE LUNG is one of the organs most commonly involved in both primary and metastatic carcinomas. Lung carcinoma is the leading cause of cancer death amongst men and is the second amongst women in industrialised countries and in many others [1].

The World Health Organisation Histological Classification of primary lung neoplasms, with the relative incidence of different histotypes, is reported in Table 1, but for clinical purposes, primary carcinomas are commonly divided into two major groups: non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) [2, 3], accounting, respectively, for around 80% and 17% of all lung tumours. Although constituted by different histological types, the NSCLCs are basically treated according to the same protocols. Radical surgery is the treatment of choice and patients are selected according to their staging. However, despite advances in diagnostic techniques, only 25% of patients with NSCLC are eligible for surgical treatment [2]. Stage is the main prognostic factor (Table 2), but other additional prognostic markers are badly needed. The main reason is that new prognostic markers could allow selection of patients with the same stage but with more aggressive or less aggressive disease, in order to offer an optimised treatment, for example, adjuvant chemotherapy in stage I patients with aggressive disease, while patients with a disease which is anatomically widespread but biologically not very aggressive could become eligible for surgery. As in other type of tumours, intratumoral microvessel density (IMD) has been recently investigated, but all papers published so far have been concerned with NSCLC. In the present paper, we review the published studies and some preliminary data available from our laboratories (Table 3).

The lung is also a very common site of metastatic disease from carcinomas, melanomas and soft tissue tumours. Metastatic carcinomas can originate from any site, the most common being breast, gastrointestinal, urogenital and head and neck. Angiogenesis is regarded as essential for tumour growth, and clinically detectable metastasis are widely regarded as being made up of angiogenic clones [4, 5]. Despite the relevant role played by angiogenesis in metastasis, we are aware of just one paper investigating IMD in human metastatic disease [6]. In this study, it was reported that IMD in brain metastatic small cell lung carcinoma is higher than in metastatic lung squamous cell carcinoma, and that a strong expression of basic fibroblastic growth factor, a potent angiogenic factor, is present in both neoplastic and glial cells.

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STUDIES INCLUDING NSCLC PATIENTS AT DIFFERENT STAGES OF DISEASE

Most of the studies published in the literature concern patients at different stages of disease (Table 3). A series of 253 NSCLC was investigated by Fontanini and associates [7]. The series contained patients with stage I–IIIB disease. The main aim of the study was to correlate IMD with the presence of node metastasis at the presentation of the disease, and indeed, high IMD in the primary tumour was associated with high incidence of metastasis in hilar and mediastinal nodes. Other parameters were also associated with node metastasis (sex, histotype, tumour size and vascular invasion), but IMD was the strongest independent marker. Follow-up was available only for 94 patients and IMD was associated with both shorter overall survival and disease-free survival, but it was dependent of node status.

Another study concerns a series of 107 patients with stage I and II disease [8]. The results reported by Fontanini were confirmed: IMD was correlated with node involvement and was independent of node status as a prognostic marker. The authors also looked independently at squamous cell carcinoma and adenocarcinoma. No evidence of a correlation between IMD and prognosis were found in the latter. However, the series was very small with only 38 cases of adenocarcinoma.

Similar data on adenocarcinoma have been reported by Yamazaki and associates [9] in a series of 42 cases (stage I–IV). No association between IMD and survival and node metastasis was found. Only a relationship between IMD and the occurrence of distant metastasis was observed.

The relationship between IMD, tumour stage, lymph node involvement and disease-free survival has also been investigated in a series of 55 NSCLC patients at various

Table 1. The World Health Organization Histological Classification of primary lung tumours

Primary lung tumours	Frequency (%)
Carcinoma	97.9
NSCLC	80.3
Squamous cell carcinoma	31.1
Adenocarcinoma	29.4
Undifferentiated large cell carcinoma	7.4
Giant cell carcinoma	0.4
Others	12.0
SCLC	16.8
Carcinoid	0.8
Others	2.1
Sarcoma	0.1
Other specified	0.1
Unspecified	1.9

Modified from [34].

stages of the disease by Yuan and coworkers [10]. Once again, high IMD correlated with advanced stage and early relapse. Furthermore, the authors reported higher IMD in adenocarcinoma, suggesting that it could be responsible for their more aggressive behaviour with respect to squamous cell carcinoma.

STUDIES INCLUDING NSCLC PATIENTS WITH DISEASE AT THE SAME STAGE

Two studies thus far have been published investigating a series of patients with the same stage of disease, both by Macchiarini and coworkers [11, 12]. In the first study [11], they investigated a series of 87 stage I (T1 N0 M0) patients looking at the incidence of metastatic disease during the follow-up. The median follow-up was 95 months for patients who relapsed, and 29 months for patients free of disease. The authors showed that the risk of relapse was directly proportional to the IMD in the primary tumour. In a multivariate analysis, IMD was the only independent factor predicting metastasis. In a second series of 28 patients, all with NSCLC invading the thoracic inlet, the relationship between IMD and survival and disease-free interval was investigated [12]. For both these parameters, univariate and multivariate analysis showed that IMD was the only prognostic factor.

Table 2. Stage and survival in NSCLC

		Survival at 5 years
Stage I	T1-2 N0 M0	60–80%
Stage II	T1-T2 N1 M0	25–50%
Stage II	T3 N0-1 M0	25–40%
	T1-3 N2 M0	10–30%
Stage IIIB	any T4 or any N3 M0	< 5%
Stage IV	any M1	< 5%

We are currently investigating a series of 500 NSCLC in stage I for several prognostic factors, among them angiogenesis. The final analysis on the whole series is still in progress, but preliminary data on the role of IMD on the first of 387 cases are presented. No significant difference either in survival or in disease-free interval between patients with higher and lower IMD (Figure 1) has been observed.

In this study, we noticed that in a minority of cases, a peculiar relationship between tumour and normal lung could be observed. While the majority of NSCLC tumours destroy and take the place of normal tissue, producing their own associated stroma and vessels, in this particular group the neoplastic cells fill the alveolar space, but no associated stroma or vessels are observed. The intratumour vessels are recognisable as the normal lung vessels because they maintain the alveolar pattern and, most of all, are frequently accompanied by anthracytic pigment, that obviously cannot be found in newly formed intratumour vessels, which are sealed from air. Our preliminary analysis, on 387 cases, has shown that patients with this type of tumour have both a shorter survival and disease-free interval (Figure 2). A more detailed analysis on all the cases of our series is in progress.

NOVEL STUDIES ON LUNG SECONDARIES

The lung is very frequently involved in metastatic disease. We present our results of a first investigation of IMD and distribution in a series of 36 lung metastases from carcinomas and melanomas. The aim of this study was to compare IMD of primary and metastatic tumours of the lung and to investigate whether, like primary tumours, metastatic

Table 3. Series of NSCLC investigated for IMD

[Ref.]	Number of cases	Diagnosis	Stage	Median follow-up in months (range)	Main result
[7]	253 (follow-up in 94)	NSCLC	I-IIIB	16 (2–41)	Higher IMD associated with node metastases
[8]	107	NSCLC	I-II	45 (2–96)	Higher IMD associated with node metastases
[9]	42	Adenocarcinoma	I-IV	71 (39–107)	Higher IMD related to distant metastases
[10]	55	NSCLC	I-IIIB		Higher IMD related to distant metastases
[11]	87	NSCLC	I		Higher IMD related to distant metastases
[12]	28	NSCLC	IIIB	42 (8–145)	Higher IMD related to earlier relapse
Pastorino, preliminary data	387	NSCLC	I	48 (1–239)	No correlation found between IMD, survival and relapse

NSCLC, non-small cell lung cancer.

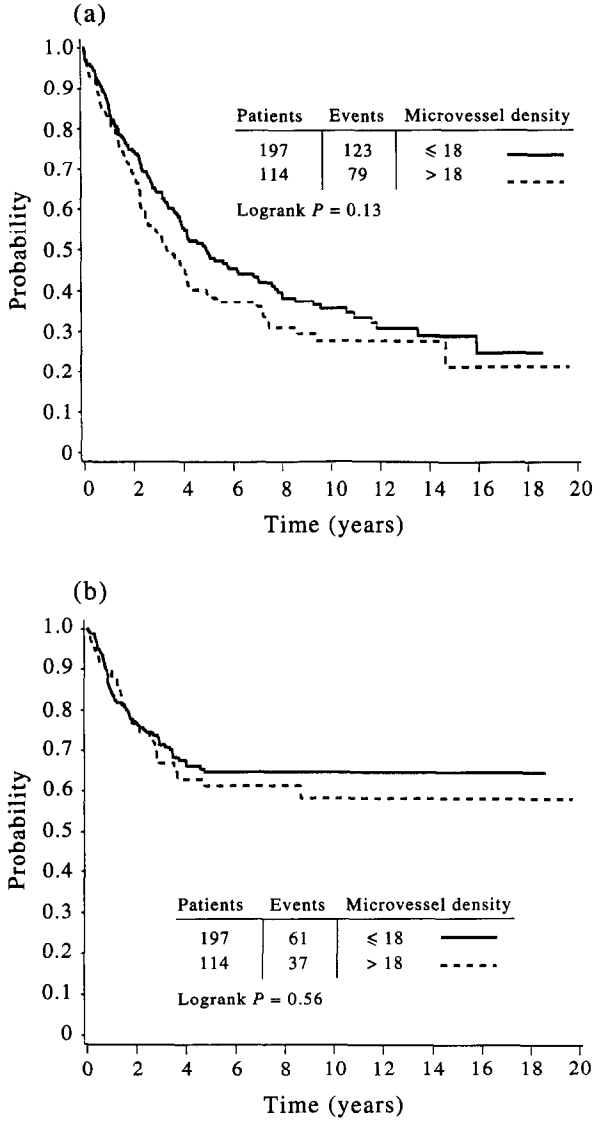

Figure 1. (a) Survival curves and (b) disease-free survival curves for stage I NSCLC patients, according to intratumour microvessel density. Microvessel density is expressed as a score by Chalkley graticule counting of the three hottest vascular spots.

tumours can also exploit lung vessels. This is an important point because it could explain why some patients have metastases despite a primary tumour with low IMD. If high IMD accounts for the metastatic capacity of many tumours, it remains to be explained how some patients can have or develop metastasis despite low IMD.

A second objective was to look at expression of some proteins involved in the regulation of apoptosis. This is another important point because it has been recently shown, in a mouse model, that dormant metastases are characterised not only by lack of angiogenesis, but also by a high rate of apoptosis [13]. Therefore, we also looked at the expression of two proteins involved in the regulation of programmed cell death: bcl-2 which prevents apoptosis [14] and p53 which induces it [15].

Methods

36 lung metastases from breast (15 cases), colon (13 cases), kidney (3 cases), ovary and adrenal (1 case each) and melanoma (3 cases) were retrieved from the files of the Department of Pathology, European Institute of Oncology, Milano, Italy and the Department of Pathology, Royal Brompton Hospital, London, U.K. 311 NSCLC were retrieved from the files of the Department of Pathology, National Cancer Institute, Milano, Italy. The diagnosis was made on conventional H&E staining. Immunohistochemistry, as previously described [16], on paraffin embedded sections, was used to examine IMD and the expression of other factors. Vessels were stained with the anti-CD-31 monoclonal antibody JC-70 [17] (courtesy of Dr D. Y. Mason) which identifies endothelial cells. Other antibodies employed were: Bcl-2 100 [18] (courtesy of Dr D. Y. Mason), anti-p53 1801 (Oncogene), anti-oestrogen receptor (ER) (Dako), anti-progesterone receptor (PgR) (Novocastra) and anti-Ki-67 antibody MIB-1 (Dianova).

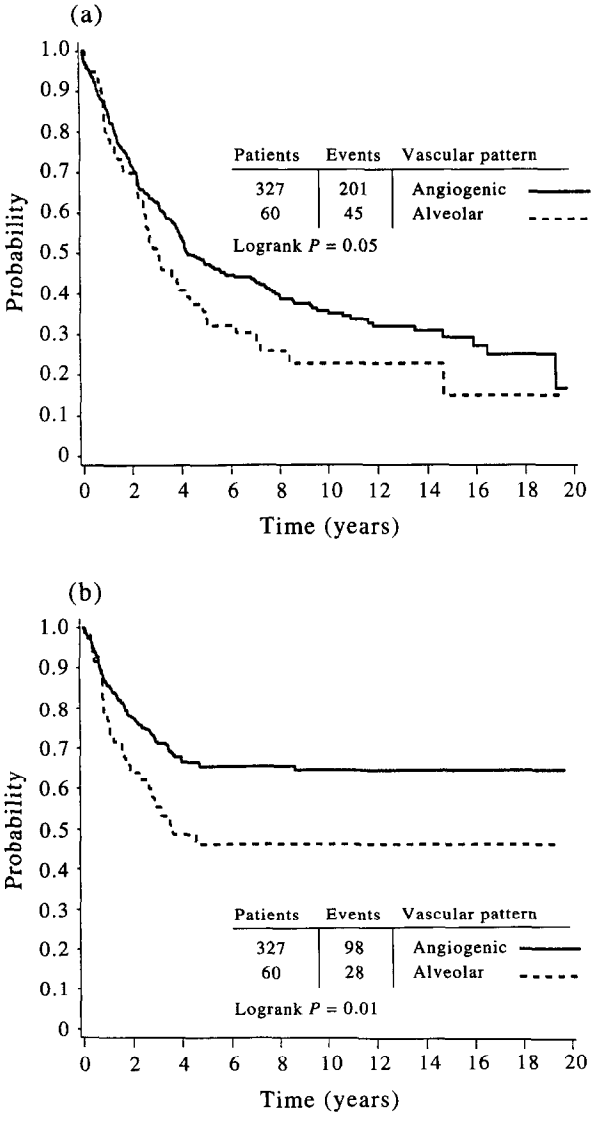

Figure 2. (a) Survival curves and (b) disease-free survival in stage I NSCLC patients, according to the vascular patterns.

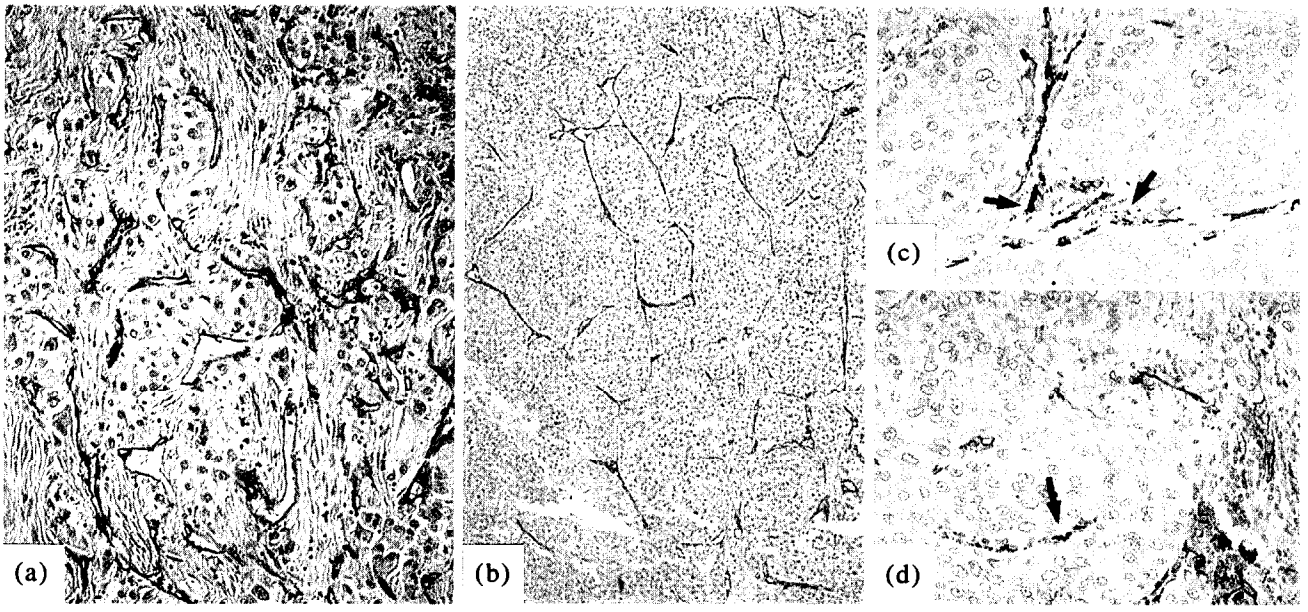

Figure 3. Vessels in lung metastatic breast carcinoma (immunostaining with the anti-CD-31 antibody JC70 immunoperoxidase technique). (a) Angiogenic pattern: no residual lung structures are observed. Vessels are randomly distributed throughout the tumour. (b) Alveolar (non-angiogenic) pattern: Immunostaining for CD-31 highlights the alveolar pattern of the lung frozen by neoplastic growth. (c,d) Alveolar pattern: Alongside the vessels, deposits of black anthracytic pigment (arrows) are present, demonstrating that these are alveolar vessels.

IMD was evaluated as previously described [19] by the use of the Chalkley camera.

IMD in metastases

In 28 out of 36 metastases investigated, lung parenchyma was replaced and the neoplastic cells were accompanied by tumour-associated stroma and newly formed vessels as commonly described, hereafter termed the angiogenic pattern (Figure 3). In the remaining 8 metastases (5 from breast and from 3 colon), a different pattern was present. The lung architecture was still present, the neoplastic cells filled the alveoli and recognisable vessels could be identified as lung vessels from their alveolar pattern of distribution and from the presence of anthracytic pigment (Figure 3), hereafter termed the non-angiogenic pattern.

The values of IMD in the metastases and a comparison with IMD in NSCLC is reported in Tables 4 and 5. IMD in metastasis was higher than in primary NSCLCs. A significant difference ($P < 0.001$) was maintained between angiogenic metastasis and primary NSCLC, but there was no significant difference between non-angiogenic metastases and primary NSCLC ($P > 0.6$).

As expected, comparison of IMD between angiogenic and non-angiogenic metastasis showed a significant difference ($P < 0.05$; Table 5).

Expression of bcl-2, nuclear p53, Ki-67 and, in metastases from breast carcinomas, ER and PgR, is reported in Table 6. Bcl-2 was expressed in 17 cases, nuclear p53 in 5 and both proteins were expressed in 5 more cases. All p53 positive cases showed nuclear accumulation. In 9 cases, immunostaining for both bcl-2 and p53 was negative. Expression of ER and PgR was investigated in 15 metastases from breast carcinoma. The 9 cases expressing either ER and/or PgR were all bcl-2 positive. Bcl-2 was also expressed in 3 out of 6 cases negative for both ER and PgR. The expression patterns of bcl-2 and p53 proteins suggest that inhibition of apoptosis could be present in 7 out of 8 metastases exploiting the lung vessels and 20 out of 28 metastases making their own vessels and stroma, assuming that the expression of bcl-2 with or without p53 expression and p53 nuclear accumulation are indicators of apoptosis inhibition.

CONCLUSION

The presence of vessels is an essential condition for neoplastic growth and this has led to the observation that the more a tumour is angiogenic, the more it is likely to give rise to metastases [4]. At the primary tumour level, there will be more cells spreading outside the tumour and the more angiogenic of those cells will be quicker in their

Table 4. IMD in lung metastases and in stage I NSCLC*

	Metastases (all)	Metastases (angiogenic)	Metastases (non-angiogenic)	NSCLC (all)	NSCLC (angiogenic)	NSCLC (non-angiogenic)
No. of cases	36	28	8	311	261	50
Mean	24.19	25.71	18.6	17.02	16.84	17.98
Standard deviation	8.79	8.93	5.66	4.75	4.80	4.40
Median	23	24	18.5	17	16	17.5
Range	12–45	13–45	12–28	5–36	5–36	10–27

*Microvessel density is expressed as a score by Chalkley graticule counting of the three hottest vascular spots.

Table 5. Comparison of IMD between 36 secondary and 311 primary NSCLCs

Tumours (No. of cases)	Mean MVD	Difference between IMD means (CI 95%)	*P* value (two-tailed)
Metastases, all (36)	24.19		
versus		7.17 (5.3–8.9)	<0.001
NSCLC, all (311)	17.02		
Metastases, angiogenic (28)	25.71		
versus		8.87 (6.7–10.9)	<0.001
NSCLC, angiogenic (261)	16.84		
Metastases, non-angiogenic (8)	18.87		
versus		0.89 (−3–4.28)	>0.6
NSCLC, non-angiogenic (50)	17.98		
Metastases, angiogenic (28)	25.71		
versus		7.11 (0.03–13.63)	<0.05
Metastases, non-angiogenic (8)	18.60		
NSCLC, angiogenic (261)	16.84		
versus		1.14 (−0.28–2.52)	>0.1
NSCLC, non-angiogenic (50)	17.98		

Table 6. Immunophenotype of 36 lung metastases

Case	Primary	bcl-2	p53	Ki-67 (%)†	ER (%)†	PgR (%)†	CD-31*	Vessel pattern‡
1	Breast	neg	pos	10	0	0	28	Non-angiogenic
2	Breast	pos	pos	60	0	0	19	Non-angiogenic
3	Breast	pos	neg	25	15	5	25	Non-angiogenic
4	Breast	pos	pos	20	70	70	23	Non-angiogenic
5	Breast	pos	neg	20	80	45	18	Non-angiogenic
6	Breast	pos	neg	10	0	0	45	Angiogenic
7	Breast	neg	neg	30	0	0	19	Angiogenic
8	Breast	pos	neg	16	0	0	29	Angiogenic
9	Breast	neg	pos	43	0	0	23	Angiogenic
10	Breast	pos	neg	17	0	15	30	Angiogenic
11	Breast	pos	neg	nd	5	27	22	Angiogenic
12	Breast	pos	neg	nd	10	38	22	Angiogenic
13	Breast	pos	neg	8	30	25	17	Angiogenic
14	Breast	pos	neg	8	30	25	17	Angiogenic
15	Breast	pos	neg	15	90	0	43	Angiogenic
16	Colon	neg	pos	75			12	Non-angiogenic
17	Colon	pos	neg	35			12	Non-angiogenic
18	Colon	neg	neg	42			14	Non-angiogenic
19	Colon	neg	neg	14			16	Angiogenic
20	Colon	pos	pos	67			32	Angiogenic
21	Colon	pos	neg	68			27	Angiogenic
22	Colon	pos	pos	53			15	Angiogenic
23	Colon	neg	neg	16			13	Angiogenic
24	Colon	neg	neg	19			19	Angiogenic
25	Colon	neg	pos	80			24	Angiogenic
26	Colon	pos	pos	60			14	Angiogenic
27	Colon	neg	neg	15			24	Angiogenic
28	Colon	neg	neg	30			28	Angiogenic
29	Kidney	neg	neg	20			27	Angiogenic
30	Kidney	pos	neg	14			37	Angiogenic
31	Kidney	pos	neg	8			28	Angiogenic
32	Melanoma	pos	neg	19			42	Angiogenic
33	Melanoma	pos	neg	27			15	Angiogenic
34	Melanoma	pos	neg	24			23	Angiogenic
35	Adrenal	neg	neg	12			38	Angiogenic
36	Ovary	neg	pos	65			31	Angiogenic

*Score by Chalkley camera counting on the three hottest spots. †Percentage of positive tumour cells. ‡Non-angiogenic pattern represents tumours exploiting normal lung vessels; angiogenic pattern represents tumours producing their own vessels and stroma.

growth [5]. Indeed, it is assumed that clinically detectable metastases are due to angiogenic clones. However, it is well known from clinical practice that metastases can appear following a long disease-free interval.

Experimental evidence now demonstrates that this late metastasis results from the progression of the so-called dormant metastases. According to a recently proposed model, based on murine studies, dormant metastases are made up of poorly angiogenic or non-angiogenic clones with a high apoptotic rate. These micrometastases are, therefore, unable to grow more than a few millimetres despite a high proliferation fraction. However, because of this high proliferation, these clones can survive for years. Only when some of the cells switch to an angiogenic phenotype and the rate of cells undergoing apoptosis decreases, can the clones start to grow, resulting in a clinically detectable metastases [13, 20].

A second feature of tumour growth is the ability to destroy surrounding normal tissue. A set of enzymes is commonly activated (e.g. urokinases, collagenases) and this degrades the normal tissue and allows expansion of the tumour and its associated stroma and vessels [21–24].

Our results confirm that high IMD is present in clinically detectable metastases. However, we report that in the lung, which provides an optimum vascular bed around empty spaces, a metastases can develop without producing either new vessels or any stroma. This is a novel finding, but in many ways the lung is the most suitable organ; not only does it offer an excellent vascular bed, but its alveolar structure allows neoplastic growth if the tumour is unable to produce remodelling of the surrounding tissue. This angiogenic-independent growth has not been previously reported and we have demonstrated that primary lung tumours exploiting local vessels are more aggressive. It will be interesting to determine whether this is also true for metastases. This type of tumour is likely to differ also in other characteristics, for example, expression of proteases.

Our data begins to provide a basis for an explanation as to why some tumours with low angiogenesis can still metastasise and why the lung is one of the preferential targets for some primary tumours. The possibility for clones with low or angiogenic capacity to give rise to clinical metastases in the lung suggests this type of disease will be increasingly seen in patients treated with anti-angiogenic drugs.

A second point we have demonstrated is that, in a high number of metastases [19–33], alongside an optimum vascularisation, the bcl-2 protein is expressed. This protein is well known for its ability to prevent apoptosis [14]. Our data support the evidence obtained in mice that the dormant metastases must also switch to expression of anti-apoptotic proteins in order to grow [13]. The second protein investigated was p53. In its wild type conformation, it is involved in the pathway inducing apoptosis [15] and we have reported that in normal tissue it is expressed in cell populations with a high apoptotic rate [25]. However, a series of events, from the occurrence of a point mutation to overexpression of the protein mdm-2, can impair the function of p53 causing a reduction in the apoptotic rate. One of the signs associated with p53 function impairment is nuclear accumulation and we found such accumulation in 10 metastases, 5 of which expressed bcl-2 as well. Therefore, in 27 out of 36 lung metastases, the bcl-2 and p53 pattern of expression suggest that apoptosis is inhibited. This finding is at variance with the trend reported in the literature that bcl-2 positive tumours are less aggressive [26]. Particularly in breast carcinoma, bcl-2 expression is reported as associated with ER expression and better prognosis [27–33], but our data suggest that bcl-2 expression seems to be an important feature of breast carcinoma progression. It is very likely that the role of bcl-2 in tumour progression changes according to which other genes are co-expressed. Furthermore, it must be borne in mind that we have investigated only solitary metastasis suitable for surgical treatment. This means that we looked at what is likely to be metastatic disease in one of its less aggressive manifestations and, therefore, not representative of the metastatic disease in general.

Results from this pilot study suggest that metastases are a more ordered type of disease than previously thought and it is possible to classify them according to a series of biological parameters, not dissimilar to those for primary tumours. Investigation of the biological characteristics of metastatic tumours will therefore be essential for two reasons:

(1) in order to identify those primary tumours with a comparable phenotype, which are likely to be highly aggressive; and

(2) to correlate biological characteristics of metastasis with clinical behaviour and response to treatment, in order to choose the right treatment for the different types of metastases and improve the care of metastatic disease.

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