Assessment of Vascular Maturation in Non-Small Cell Lung Cancer Using a Novel Basement Membrane Component, LH39: Correlation with p53 and Angiogenic Factor Expression

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ABSTRACT

Angiogenesis, the formation of new vessels, has been demonstrated to be a potent and independent indicator of prognosis in non-small cell lung cancer patients. The extent of differentiation of the tumor vessels may affect access of peripheral white cells and egress or invasion of tumor cells. This has not been assessed in relation to tumor microvessel density or other variables and may be a marker of vascular remodeling. LH39 is a monoclonal antibody recognizing an epitope located at the lamina lucida of mature small veins and capillaries but not in newly formed vessels. We examined the ratio of mature/immature vessels in 81 non-small cell lung carcinomas and correlated the vascular maturation index (VMI) to different clinicopathological variables including angiogenesis. Mature vessels were defined by staining with antibodies to both LH39 and to CD31, using double immunohistochemistry, whereas immature vessels stained only for CD31. VMI was defined as the percentage fraction of mature vessels (LH39 positive)/total number of vessels (CD31 positive). The median VMI in lung carcinomas was 46% (range, 15–90%). There was a significant inverse correlation between high VMI and low thymidine phosphorylase expression (P = 0.0001), high VMI and nuclear p53 negativity (P = 0.01), high VMI and low angiogenesis (P = 0.0001), as well as between high VMI and absence of nodal involvement (P = 0.01). Low angiogenesis and high VMI were associated with a significantly better outcome (P = 0.0001 and P = 0.02, respectively). These findings show that there is a wide variation in the differentiation of tumor vasculature in lung carcinomas, and VMI gives new information on the degree of active tumor vascular remodeling independently from microvessel quantitation.

INTRODUCTION

The formation of new vessels, angiogenesis, is essential for tumor growth and metastasis (1). The angiogenic process includes retraction of pericytes, protease dissolution of the capillary basement membrane and extracellular matrix, and EC migration. Sprouts are formed that elongate and fuse to form a network of interconnecting loops (2). Capillary differentiation, including the formation of an irregular basement membrane composed of abnormal ratios of the normal constituents, then occurs, before initiation of the blood flow (3, 4).

LH39 is an antibody to a basement membrane epitope located in the lamina lucida (5). Normal basement membranes are complex, highly compartmentalized structures, composed of three major structural zones (lamina lucida, lamina densa, and lamina reticularis; Ref. 6). Tumor vasculature is known to consist of an irregular basement membrane, composed of variable amounts of fibronectin, laminin, and collagen, depending on the maturation status of the capillary (4). Such structural defects of the microvasculature, together with the functional effects of various angiogenic factors, account for the permeability of the tumor-associated endothelium. LH39 has recently been demonstrated to be present in the basement membranes of small veins and capillaries within the stroma of normal human organs but was absent in newly formed vessels of several pathological conditions (e.g., ulcers and granulomas) and in cancer (5, 7). LH39 was also found to be absent in large and medium-sized arteries and veins of normal, inflammatory, or neoplastic tissues (7). This is of additional interest, because other basement membrane compounds, like laminin and collagen type IV, are known to be expressed in vessels of all sizes.

The formation of a new basement membrane usually comes as a late event, after migration and proliferation of ECs within the sprout (8). The establishment of a mature basement membrane has been shown to be accompanied with restricted EC proliferation, something that is mostly associated with the presence of pericytes in the vessel wall (9). Hence, although EC proliferation is required for angiogenesis, it is the migration and remodeling of the existing tissue vascular supply that establish the functional vasculature within the tumor (10).

In the present study, we examined the VMI in lung carcinomas by measuring the proportion of the total tumor vasculature highlighted by the EC marker CD31 and the basement membrane component LH39. This ratio will give the proportion of vessels in individual tumors that are undergoing remodeling and are still in an immature state. TP, also known as platelet-derived endothelial cell growth factor, has been shown to exhibit a chemotactic and mitogenic capacity on endothelial cells in several angiogenic model systems (11–13). In lung carcinomas, TP overexpression has been correlated with high angiogenesis and poor prognosis (14). VEGF, also known as vascular permeability factor, has been shown to render vessel hyperpermeability, to be a potent mitogen for endothelial cells, and to promote tube-like vessel structure formation and vessel maturation (15). In NSCLC, VEGF overexpression was found to correlate with bad prognostic features such as nodal involvement and poor survival (16). An association between VEGF and p53 expression was also observed in lung carcinomas (16). Therefore, we have also evaluated the expression of VEGF, TP, and p53 and have compared all of these measurements to conventional clinicopathological variables and quantitative angiogenesis.

MATERIALS AND METHODS

Patients and Tumors

Freshly frozen samples of normal lung specimens (n = 8) and from 81 patients undergoing resection for primary lung cancer were obtained from Cellular Pathology (John Radcliffe Hospital, Oxford, United Kingdom). The specimens were snap frozen in liquid nitrogen and stored at −70°C. Cryostat sections (7-μm thickness) were fixed in acetone for 10 min at room temper-
ature, left to dry overnight, and either stained immediately or stored at \(-20^\circ C\) until required. Formalin-fixed, paraffin-embedded tissue samples from the same lung tumors were also cut in 4-μm-thick sections and mounted onto silane-coated slides. Patients were T_{1/2} and N_{0/1} pathologically staged, corresponding to stages I and II of the International Staging System for Lung Tumors. They were treated with surgery alone and had survived at least 60 days after operation, to exclude perioperative mortality-related bias. No chemotherapy or radiotherapy was given before surgery, which represented the sole mode of treatment. During follow-up, 43 patients died. The 38 patients alive at the time of the study had been followed up for a median of 53 months (range, 35–83 months). Histological diagnosis and grading and nodal status assessment were performed on H&E-stained sections. Fifty-one (63%) patients had squamous cell carcinomas, and 30 (37%) had adenocarcinomas. Lymph node involvement was present in 29 (36%) patients. Histological grade I/II was confirmed in 34 (42%) cases, and grade III was confirmed in 47 (58%).

**Immunohistochemistry**

Antibodies. Lamin A, lumina antigen, LH39 (supematant), was kindly provided by Prof. I. Leif (London, United Kingdom; Ref. 5). Antibodies for PECAM/CD31 (JC70A), TP/PG44c (supematant), p53 (CM-1), and VEGF (VGI) were provided by Prof. K. C. Gatter (Oxford, United Kingdom; Refs. 16–19).

LH39/CD31. Immunohistochemistry was performed on cryostat sections mounted onto multiwell slides. Each slide contained four sections (7-μm thickness). One of them was used for double CD31/LH39 staining, two for single CD31 and LH39 staining, respectively, and the remaining one was used as a negative control by replacing the primary antibody with a nonspecific IgG antibody. For double staining, sections were incubated with LH39 overnight in a moist chamber at 4°C. Secondary antibody, biotinylated goat anti-mouse immunoglobulin (DAKO; K492) was applied for 20 min, followed by incubation with streptavidin biotinylated horseradish peroxidase for another 20 min. Peroxidase reaction was developed using diaminobenzidine tetrahydrochloride (Sigma). Slides were then incubated with JC70 (CD31) for 1 h at room temperature before sequential application of anti-mouse immunoglobulin (DAKO; Z239, at 1:50 dilution) and APAAP complex for 30 min each. This step was repeated twice (second and third incubations) for an additional 10 min each to enhance the intensity of the final staining. The APAAP reaction was visualized using new fuchsin (DAKO; K596), and slides were weakly counterstained with hematoxylin and mounted in aqueous medium. Three 5-min washings between incubations in Tris-buffered saline (TBS; pH 7.2) was performed.

Other Immunohistochemical Techniques. The horseradish peroxidase method was performed to visualize TP staining on paraffin-embedded tissue samples, with PG44c monoclonal antibody as primary, as described previously (18). Assessment of TP expression was based on both the intensity of staining and the extent of staining, with four grades: (a) the tumor cells; (b) stroma cells (overall TP; Ref. 20). The percentage of cancer cells with strong TP reactivity was recorded. Cases were considered as bearing strong TP reactivity if >50% of cells displayed strong staining. Strong TP reactivity in the stroma in >50% of the optical fields was used as cutoff point to define cases with low or high stroma TP reactivity (20).

VEGF expression was assessed on paraffin-embedded tissue samples using the VGI monoclonal antibody (recognizing the 121-, 165-, and 189-amino acid isoforms of VEGF) and the horseradish peroxidase method with microwave retrieval, as described previously (16). The percentage of cancer cells with positive reactivity was recorded, and three groups were considered, based on the extent of positive staining (low, 0–29%; intermediate, 30–70%; and high, >70%; Ref. 16).

p53 expression was detected on cryostat sections using the CM-1 polyclonal antibody. Samples with positive staining in <10% of tumor cells were considered negative, as described previously (19). In all cases, the specificity of the staining was confirmed by replacing the primary antibodies with an irrelevant isotype antibody.

**Quantitation of Tumor Angiogenesis**

Microvessel counting was used for angiogenesis assessment. Sections were scanned at low power (×40 and ×100) to choose the areas of the highest vascularization and microvessel counting, followed at ×200 power on three chosen fields of the highest density. The MS was the sum of the vessel counts obtained in these three fields. Only vessels with a clearly defined lumen or a well-defined linear shape but not single endothelial cells were taken into account for microvessel counting. High vascular grade was defined as a MS of >75, whereas low vascular grade was defined as a MS of <75, as described previously (21).

**VMI**

All vessels were stained with anti-CD31 antibody, but only a subset stained for LH39. Assessment for both CD31 and LH39 was done at the tumor stromal interphase. VMI was defined as the percentage of LH39 stained to CD31-stained vessels. VMI was assessed in two different ways using the score obtained from either the single-stained or the double-stained sections on the multwell slides. The index in double-stained sections was derived by counting the total number of double-stained (LH39 + CD31) and single-stained (CD31) vessels in each tumor section (vessel counting was performed in the same three hot spot areas selected previously for angiogenesis assessment). Thus, the index was LH39-positive vessels/total CD31-positive vessels. The VMI was also calculated separately, in a similar way, using the microvessel score obtained from LH39 and CD31 single staining. The VMI was determined independently by two observers.

**Statistics**

Statistical analysis was performed using Graph Pad Prism 2.01 and Instat 2.0 Computer Packages. Fisher’s exact test, unpaired or paired two-tailed t test was used to examine the relationship between categorical variables, as appropriate. Linear regression analysis was performed to assess interobserver variability and to evaluate correlation between continuous variables. The log-rank test was used to perform univariate survival analyses, whereas multiple regression analysis was used for multivariate models. P < 0.05 was used for significance.

**RESULTS**

Double staining for tumor vessels with antibodies to CD31 and LH39 antigen is shown in Fig. 1. All LH39-positive vessels identified throughout the tumor body were also positive to CD31. However, a varying proportion of CD31-positive vessels were negative for LH39. Linear regression analysis of the VMI obtained from single and double staining showed statistically significant association (P < 0.0001, r = 0.88). Interobserver variability was also minimal for both single staining (P < 0.0001, r = 0.93) and double staining (P < 0.0001, r = 0.82), respectively. Discrepancies were resolved by consensus over a conference microscope.

**VMI Assessment and Relationship to Other Histopathological Parameters**

In the eight normal lung cases examined, the median VMI value was 82% (range, 75–95%). The median VMI in the 81 lung carcinomas examined was 46% (range, 15–90%). VMI expression was analyzed either as a continuous or as a categorical variable. On splitting VMI into thirds (33 and 66% percentiles; cutoff points, 34 and 50%), we categorized VMI in lung carcinomas as high, medium, or low. The unpaired two-tailed t tests performed to assess relationships of VMI to different histopathological tumor variables revealed a significant association between high VMI and absence of nodal involvement (P = 0.01), as well as between high VMI and low microvessel score assessed with CD31 (P = 0.01; Table 1). No significant associations between VMI and other tumor parameters were observed (Table 1). In a bivariate model, we found that only the vascular grade was independently associated with nodal involvement (P = 0.02, t = 2.3), whereas VMI was not (P = 0.09, t = 1.7). The number of LH39-positive vessels was not associated with any of the parameters examined (data not shown).

**VMI Correlation with Angiogenic Factors and p53**

The patterns of immunoreactivity for TP/platelet-derived endothelial cell growth factor in NSCLCs have been described in a previous study...
Immunostaining was assessed in cancer cells and stroma (macrophages and fibroblasts) cells. Strong TP reactivity in >50% of cancer cells was observed in 32 of 81 cases, whereas strong stroma TP reactivity was observed in 16 of 81 cases.

The pattern of VEGF staining was granular cytoplasmic, as described previously (16). Tumor cells were positive for VEGF in 44 of 81 cases with lung carcinomas. Tumor stromal fibroblasts, macrophages, and blood vessels were only occasionally positive. Forty-five of 81 cases were positive for p53 nuclear accumulation, whereas 36 of 81 were negative.

A strong association of high VMI with low TP reactivity ($P = 0.0001$) and low p53 nuclear accumulation ($P = 0.01$) was observed (Table 1). No association was found for VEGF groups. The linear regression analysis performed revealed significant inverse associations between VMI and vascular grade ($r = 0.40, P = 0.0001$; data not shown), VMI and TP ($r = 0.43, P = 0.0001$; Fig. 2), and VMI and nuclear p53 reactivity ($r = 0.26, P = 0.01$; data not shown) but not between VMI and VEGF. Combined expression patterns of TP and p53, according to their negative or positive expression, were produced and examined for relationships to VMI using unpaired $t$ tests. TP negativity was significantly associated with high VMI ($P = 0.0001$; Fig. 3). Similarly, combined expression patterns of VEGF and TP were produced, and again TP negativity was significantly associated with a higher VMI ($P = 0.0007$; Fig. 4).

**Survival Analysis.** The univariate analysis revealed that angiogenesis was a strongly significant prognostic indicator for survival in this group of patients ($P = 0.0001$; Fig. 5A). Patients with high or median VMI had a significantly better prognosis as compared with those having a low VMI ($P = 0.02$ and $P = 0.03$, respectively; Fig. 5B). Vascular grading using the LH39-positive vessels was not related to prognosis (data not shown). Subgroups combining angiogenesis with different VMI categories were also produced, and VMI was found to have no impact in defining subgroups with different prognosis in low or high angiogenesis cases. Cases with low angiogenesis had a statistically better prognosis as compared with cases with high angiogenesis, regardless of VMI status ($P = 0.0001$; Fig. 5C). In bivariate analysis, only the VG was an independent prognostic variable ($P = 0.0005$; hazard ratio, 3.63)

**DISCUSSION**

Although most solid tumors are highly vascular, tumoral vessels are not identical to normal vessels in mature tissues. The distinction between tumor and normal blood vessels includes differences in the basement membrane composition, differences in permeability, and...
LH39 as Indicator of Vascular Maturation in Lung Cancer

Table 1 Unpaired two-tailed t test examining relationship between VMI and other clinicopathological variables (n = 81)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value %</th>
<th>+SD</th>
<th>95% CI*</th>
<th>P</th>
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<tr>
<td>T stage</td>
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<tr>
<td>T1 (n = 28)</td>
<td>47</td>
<td>17</td>
<td>0.41–0.54</td>
<td>0.36</td>
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<tr>
<td>T2 (n = 53)</td>
<td>44</td>
<td>18</td>
<td>0.39–0.45</td>
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<tr>
<td>N0 (n = 52)</td>
<td>49</td>
<td>18</td>
<td>0.44–0.54</td>
<td>0.07</td>
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<tr>
<td>N1 (n = 29)</td>
<td>38</td>
<td>16</td>
<td>0.32–0.45</td>
<td>0.07</td>
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<tr>
<td>Grade</td>
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<tr>
<td>1/2 (n = 34)</td>
<td>49</td>
<td>20</td>
<td>0.42–0.56</td>
<td>0.07</td>
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<tr>
<td>3 (n = 47)</td>
<td>42</td>
<td>15</td>
<td>0.37–0.47</td>
<td>0.07</td>
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<tr>
<td>VEGF</td>
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<tr>
<td>Positive (n = 44)</td>
<td>43</td>
<td>16</td>
<td>0.38–0.48</td>
<td>0.36</td>
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<tr>
<td>Negative (n = 37)</td>
<td>47</td>
<td>20</td>
<td>0.40–0.54</td>
<td>0.001</td>
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<td>TP</td>
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<tr>
<td>Positive (n = 32)</td>
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<td>15</td>
<td>0.30–0.41</td>
<td>0.001</td>
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<tr>
<td>Negative (n = 49)</td>
<td>51</td>
<td>17</td>
<td>0.46–0.56</td>
<td>0.07</td>
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<tr>
<td>Vascular grade</td>
<td></td>
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<tr>
<td>High (n = 17)</td>
<td>35</td>
<td>12</td>
<td>0.29–0.42</td>
<td>0.01</td>
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<tr>
<td>Low (n = 64)</td>
<td>40</td>
<td>18</td>
<td>0.43–0.52</td>
<td>0.07</td>
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<tr>
<td>p53</td>
<td></td>
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<tr>
<td>Positive (n = 45)</td>
<td>41</td>
<td>17</td>
<td>0.35–0.46</td>
<td>0.01</td>
</tr>
<tr>
<td>Negative (n = 36)</td>
<td>50</td>
<td>18</td>
<td>0.44–0.57</td>
<td>0.07</td>
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* CI, confidence interval.

A tumor is considered to have “switched” to an angiogenic phenotype when it alters the balance of angiogenic stimulators and inhibitors, thereby gaining the capacity to establish a blood supply. Tumor angiogenesis is not an ordered process, and individual tumors vary in their ability to form structurally normal vessels (24–26). Thus, the number of tumor vessels alone will only give one aspect of the ability of a tumor to complete the complex angiogenic program. Although high VMI was associated with absence of regional node spread and significant inverse association between high VMI, low angiogenesis, and absence of nodal involvement was seen. The immature vessels are more likely to facilitate tumor cell escape into the adjacent lymphatics and may contribute via proteases to local tumor invasion. This process is more likely to be retarded in more mature vessels and thus the correlation between high VMI and absence of nodal involvement. Although VMI measures a different aspect of angiogenesis to vessel number, because it gives the proportion of tumor vessels being remodeled, this association suggests that vessel maturation and tumor angiogenic status are controlled and simultaneously regulated by similar factors. However, cases with low or median VMI were found in both high and low angiogenesis groups, suggesting that there are differences in the pathways controlling differentiation and proliferation. Thus, TP was strongly associated with a poorly differentiated vasculature, but there was no association of VEGF with VMI. TP may be synergizing with VEGF, one causing migration and remodeling and the other proliferation.

Differences in vessel stability (3). In the present study, using a double immunohistochemical labeling technique for endothelial cell and basement membrane markers, using a basement membrane component absent from vessels actively involved in neovascularization (5, 7), we have demonstrated that the degree of vascular maturation in lung tumors varies widely. Endothelial cell remodeling, besides proliferation, might be an important mechanism by which human tumors establish a blood supply (10). Thus, VMI may represent the aggregate of new vessels produced by both division and remodeling. The remodeling of tumor vasculature continues after its formation (22), reflecting a potential stratagem by which a tumor might match its blood supply to its growing demands.

An association between high VMI and low TP expression was observed in our study. TP, although not a classic growth factor, has been shown to be both chemotactic and mitogenic for endothelial cells and angiogenic in several model systems (11, 12, 23). We have shown previously in NSCLC an association between high TP expression with high angiogenesis and poor prognosis (14). Therefore, low expression of a mitogenic/angiogenic factor, such as TP, may indicate a less aggressive tumor phenotype. This would possibly facilitate the establishment of a well-differentiated/mature tumor vasculature, which by turn associates with good prognostic features, such as low angiogenesis and regional lymph node negativity. Indeed, in our cases, a
better prognosis, tumors that have acquired the ability to form mature vessels, i.e., have a high VMI similar to normal tissues, might be those tumors with a more effective blood supply. Thus, VMI might not only give an index of the proportion of vessels produced by cell division and remodeling (angiogenic index) but might also give an indication as to the function of such vessels. Indeed, particular vascular patterns in lung carcinomas (27) and ocular melanomas (28), which similarly might reflect different qualitative angiogenic phenotypes, have been shown to be associated with differences in prognosis. Additionally, VMI may be of importance in defining the degree of tumor oxygenation and drug supply that affects response to both radiotherapy and chemotherapy.

High VMI was also found to associate with p53 negativity. p53 negativity is mostly relevant to the presence of the unstable wild-type p53 protein, usually not detected by immunohistochemistry (29). However, in most studies, the prognostic role of immunohistochemical p53 expression in NSCLCs has been controversial. In our study, the observed correlation between high VMI and nuclear p53 negativity may be relevant to the role of thrombospondin, a potent angiogenesis inhibitor (30). Thrombospondin, a wild-type p53-induced protein, by down-regulating angiogenesis and keeping the vasculature in a quiescent state may indirectly facilitate the establishment of a well-differentiated vasculature.

Presently, tumor angiogenesis is measured by estimating the number of immunohistochemically identified vessels in angiogenic hot spots; however, not all studies have confirmed the utility of this technique (31). Furthermore, the efficiency of the tumor vasculature will be determined not only by the number but by the quality of the vessels generated. These limitations have encouraged the evaluation of other indirect measures of tumor angiogenesis, such as serum growth factor levels from cancer-bearing patients (32). The concept of an angiogenic profile encompassing several angiogenic pathways might be more useful, and measures of capillary maturation should be considered as a potential part. This might become more important with the advent of antiangiogenic agents directed against particular processes involved in vascular remodeling, such as endothelial cell migration, cell-matrix interactions, and synthesis of basement membrane (33–38).

These observations are also relevant to the efficacy of vascular targeting (34). This type of therapy, which aims to accurately destroy tumor vasculature with a different phenotype to normal tissues, may be even more effective than expected if such a high proportion of the vasculature is in an immature state. Recent demonstration of tumor regression with growth-inhibitory peptides specific for vascular endothelium was hard to explain if only proliferating endothelial cells were inhibited (35). However, if a large proportion of vessels are poorly differentiated, as a result of remodeling and endothelial growth, inhibition of these pathways will be more effective than expected previously.

In summary, this study has demonstrated a method for assessing maturation of tumor-associated vessels that are poorly differentiated and may therefore be involved in vascular remodeling. We also suggest that measures of capillary maturation might be complementary to microvessel number to aid the identification of patients who might benefit from specific antiangiogenic therapies or vascular targeting treatment.

REFERENCES

Fig. 5. A. univariate analysis showing a significant association between LVG and better survival. B. univariate analysis showing a significant association between HMI/MMI and better survival. C. univariate analysis of survival in subgroups of patients according to combined angiogenesis and VMI categories. LVG cases were associated significantly with better survival regardless of VMI status. LVG, low vascular grade; HVG, high vascular grade; HMI, high maturation index; MMI, median maturation index; LMI, low maturation index.
LH19 AS INDICATOR OF VASCULAR MATURATION IN LUNG CANCER


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