Introduction
Angiogenesis is believed to be a turning point in the development of solid tumours. During the avascular phase, the growth of the tumour is limited by the rate at which nutrients and metabolites diffuse across the tumour boundary, and, as a result, are typically only a few millimetres in radius. Following angiogenesis, the tumour has its own vascular network, which provides access to a limitless supply of resources and permits unlimited growth of the tumour. The vasculature also provides a route for tumour cells that break free to metastasize. Thus, angiogenesis marks a tumour's transition from a localized lesion to a systemic and potentially fatal disease [1].

Cancer-induced angiogenesis is a result of increased expression of angiogenic factors, such as vascular endothelial growth factor (VEGF), or decreased expression of antiangiogenic factors, or a combination of both events [2].

Over the last decade, assessment of angiogenesis has emerged as a potentially useful biological prognostic and predictive factor in solid human tumours [3]. Mean microvessel density (MVD) has been proven to be a powerful and often independent prognostic indicator for many different types of human cancers [4–6].

In addition, a significant correlation between the expression of VEGF and prognosis has been described [7,8]. Studies indicate that the levels of angiogenic factors in tissue reflect the aggressiveness with which tumour cells spread and thus have predictive value in the identification of high-risk patients with poor prognosis. Yet, the results of studies on tumour angiogenesis and the relationship between clinicopathological factors and prognosis in head and neck tumours, particularly in squamous cell carcinoma of the larynx (LSSC), are controversial [8–15].

Aim of the work
The present study aimed to evaluate the significance of angiogenesis in Egyptian patients with LSSC and correlate the levels of expression with clinicopathological parameters and prognostic associations.
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Patients and methods

This was a prospective study conducted at Ain Shams University Hospitals (Otorhinolaryngology and Pathology Departments) between 2006 and 2012 on 80 patients. All patients scheduled for surgical excision of laryngeal cancer were eligible for inclusion in the study. Exclusion criteria included the presence of inoperable or recurrent tumour, previous chemotherapy or radiation of the neck, and presence of end-stage organ disease such as chronic renal failure or liver cell failure. Approval was obtained from the ethical and moral committee of the Faculty of Medicine, Ain Shams University, for the design and protocol of the study, which complied with the Helsinki Declaration of 1975, as revised in 2008, and informed written consent was obtained from all patients before enrolment.

All patients were subjected to extensive workup including history taking, general and otolaryngological examination, preoperative evaluation including computed tomography scan of the neck and chest, abdominal sonography, direct laryngoscopy for biopsy, and histopathological confirmation of tumour. TNM staging of the tumour was also carried out.

After definitive surgery (partial or total laryngectomy), routine histopathological examination of specimens was carried out using haematoxylin and eosin stains. Slides were examined to confirm the diagnosis of LSSC and histopathological grading was done. In addition, specimens were immunostained for CD34 antibody using streptavidin–biotin peroxidase. Immunological detection was achieved with the commercially available monoclonal antibody 133, which stains CD34 antigen on endothelial and haematopoietic cells (Ab no. 353M; BioGenex, Biogenex Fremont, CA, USA). The super-sensitive antibodies had been optimally diluted by BioGenex and were ready for use without further dilution. Also, horseradish peroxidase kits (DAB kits) were prepared as described by the manufacturers.

The mean microvessel count of the five most vascular areas was taken as MVD1, which was expressed as the absolute number of microvessel per 0.74 mm² (×200) field.

All patients were followed up clinically for detection of any recurrence at 3 and 6 months and at 1 and 2 years after laryngectomy by means of a postoperative computed tomography scan of the neck and by direct laryngoscopy for those with partial laryngectomy at 3 months postoperatively; assessment of MVD was carried out in those with recurrent tumour (MVD2).

Statistical analysis

All data were entered and processed using SPSS (version 17; SPSS Inc., Chicago, Illinois, USA) using the Mann–Whitney U-test, the Wilcoxon signed-rank test and analysis of variance.

Results

After applying the inclusion and exclusion criteria, 80 cases were included in this study. The age of the patients ranged from 43 to 79 years (mean 59.9 ± 8.7 years). All of them were male. Thirty (37.5%) patients presented with supraglottic carcinoma, 48 (60%) with glottic carcinoma and two (2.5%) with subglottic carcinoma. As regards TNM classification, four (5%) patients presented with T1, 36 (45%) with T2, 30 (37.5%) with T3 and 10 (12.5%) with T4 stage. Seventy-four (92.5%) patients were in N0, two were in N1 (2.5%) and four (5%) were in N2 stage. All patients were in M0 stage.

Seventy-four (92.5%) patients presented with conventional LSSC; four (5%) patients had verrucous

Figure 1

CD34-positive blood vessels in squamous cell carcinoma, grade II (immunostaining, ×200).
variant and two (2.5%) patients had papillary variant. As regards the pathological grading, 26 (32.5%) patients were of grade I, 38 (47.5%) were of grade II and 16 (20%) were of grade III.

The MVD1 for patients with glottic carcinoma ranged from 13.25 to 37.6 (mean 28.63 ± 6.539) and that for supraglottic cases ranged from 32 to 87.2 (mean 49.6 ± 16.139). There was a highly statistically significant difference among subgroups according to the site of cancer (Table 1).

The MVD1 for patients in T1 stage ranged from 27.6 to 32.8 (mean 30.2 ± 3.677), that for patients in T2 stage ranged from 13.3 to 60.3 (mean 32.48 ± 10.42), that for patients in T3 stage ranged from 19.3 to 87.2 (mean 44.31 ± 19) and that for patients in T4 stage ranged from 17.2 to 40.7 (mean 28.9 ± 9.95). There was no statistically significant difference among subgroups in relation to the T stage (Table 2).

The MVD for the six cases that experienced nodal metastasis ranged from 51.2 to 87.2 (mean 73.86 ± 19.73) and for the 74 cases that did not experience nodal metastasis the MVD ranged from 17.2 to 60.3 (mean 33.39 ± 9.87), with a highly statistically significant difference (Table 3).

The MVD1 for patients with conventional squamous cell carcinoma ranged from 13.25 to 87.2 (mean 37 ± 15.9), that for the papillary variant ranged from 30 to 36 (mean 33 ± 3) and that for the verrucous variant ranged from 30 to 33.6 (mean 31.8 ± 2.5). There was no statistically significant difference among subgroups with respect to pathological variants (P = 0.052).

The MVD1 for patients with grade I carcinoma ranged from 13.25 to 51.2 (mean 29.9 ± 11.3), that for grade II ranged from 22 to 83.2 (mean 40.3 ± 17.3) and that for grade III ranged from 32.8 to 87.2 (mean 47.1 ± 20). A highly statistically significant difference was found among subgroups with respect to pathological grading (Table 4).

After 2 years of follow-up, 16 (20%) patients were seen to have developed recurrence: two (12.5%) patients developed recurrence after 6 months, 12 (75%) patients after 1 year and two (12.5%) patients after 1 year. As regards the site of recurrence, 12 (75%) patients developed in the stoma, two (12.5%) in the lymph nodes and two (12.5%) in the larynx itself (surgery was primarily cordectomy). The initial MVD (MVD1) for the 64 patients who did not experience recurrence ranged from 13.25 to 87.2 (mean 49.956 ± 26.91), with no statistically significant difference (Table 5).

On comparing MVD1 with MVD2 (the mean vascular density after recurrence) in the 16 recurrent cases, the following was found: MVD1 ranged from 13.25 to

| Table 1 Correlation between microvessel density 1 in relation to the site of laryngeal cancer |
|----------------------------------------|----------------|----------------|
| Sites                    | MVD1 Range     | Mann–Whitney U-test |
| Glottic                  | 13.25–37.6     | 28.63 ± 6.539    | Z = –4.908, P-value < 0.001* |
| Supraglottic             | 32–87.2        | 49.6 ± 16.139    |                  |
| MVD, microvessel density; *Statistically significant. |

| Table 2 Correlation between microvessel density 1 in relation to T stage |
|---------------------------------|----------------|----------------|
| MVD1 Range                      | Mean ± SD     | ANOVA F-value |
| T1 27.6–32.8                    | 30.200 ± 3.677| 2.591, 0.068   |
| T2 13.3–60.3                    | 32.486 ± 10.242| 2.591, 0.068   |
| T3 19.3–87.2                    | 44.313 ± 19.072| 2.591, 0.068   |
| T4 17.2–40.7                    | 28.900 ± 9.952| 2.591, 0.068   |
| ANOVA, analysis of variance; MVD, microvessel density. |

| Table 3 Correlation between microvessel density 1 in relation to nodal metastasis |
|-------------------------------------|----------------|----------------|
| MVD                               | MVD1 Range     | Mann–Whitney U-test |
| MVD of nodal metastatic cases (n = 3) | 51.2–87.2     | 73.86 ± 19.73    | Z = 2.747, P-value < 0.001* |
| MVD of nonmetastatic cases (n = 37)  | 17.2–60.3      | 33.39 ± 9.87     |                  |
| MVD, microvessel density; *Statistically significant. |

CD34-positive blood vessels in squamous cell carcinoma, grade III (immunostaining, ×200).
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Discussion
Theoretically, angiogenesis should be a poor prognostic factor in neoplasms as it allows the tumour to acquire new blood vessels supplying nutrients to tumour cells; it is also thought to be a way for tumour cells to metastasize, turning a local disease into a systemic one. Yet, conflicting evidence exists about the role of angiogenesis and the importance of MVD as a prognostic factor in head and neck squamous cell carcinoma (HNSCC). According to many reports [13,17,18], high MVD was associated with metastasis and/or a worse clinical outcome. Moreover, VEGF expression was reported to correlate with tumour differentiation and stage [8,19]. Also, a correlation with lymph node metastases was shown [8,20]. However, other reports [15,21–23] failed to demonstrate such a relationship.

The purpose of this study was to clarify the relationship between the degree of angiogenesis and clinical and prognostic parameters in patients with LSCC, which may lead to the application of promising antiangiogenesis therapy to patients with LSCC. The degree of angiogenesis was determined by quantifying the percentage of CD34-related antigen immunostaining of vascular endothelial cells found in laryngeal tumour specimens.

We found a statistically significant correlation between MVD and occurrence of regional nodal metastasis, which is considered to be the most important prognostic index in HNSCC. This agrees with the theory that tumours with more angiogenesis have greater tendency to metastasize and therefore have a poorer prognosis, which was also observed by other reports [9,24]. In contrast, other studies [12,25] did not show such a statistical correlation; yet, they stated that there was a ‘tendency’ for an increased degree of angiogenesis in tumours with metastases in regional lymph nodes.

A highly statistically significant correlation was found between MVD and the site of tumour, as supraglottic tumours, known to have a higher incidence of nodal metastasis and poorer prognosis, were found to have a statistically significantly higher MVD compared with glottic tumours, which are known to have better prognosis. Similar results were reported by Do et al. [23]; however, they were not reproducible [11,13].

Further, in our study a positive correlation was found between MVD and pathological grade of the primary tumour, with some studies [8,19,25] confirming this result and others [15,20,24] failing to demonstrate such a relation. The increased MVD in less-differentiated tumours in our study suggests that the increase in MVD may be related to loss of differentiation. This may also be a cause for the greater aggressiveness of less-differentiated tumours.

The results of our study failed to demonstrate that MVD is a predictor of recurrence in LSSC. Yet, when comparing the initial MVD with MVD after recurrence for the 16 patients who experienced recurrence (MVD2), a statistically highly significant difference was found. This may be because recurrent tumours are usually more aggressive. Yet, it casts a shadow on the possibility of using MVD as a prognostic factor for recurrence. This result was also reported by Hagedorn and Nerlich [11], Pietruszewski et al. [13], and Pignataro et al. [26], although other studies [9,14,27,28] have shown contradictory results.

The causes of these contradicting results can be attributed to the fact that direct methods for measuring angiogenic activity in humans are lacking, which is a major difficulty in studying angiogenesis. The relationship between MVD and prognostic parameters in HNSCC could not be completely explained [29]. Although it is difficult to measure angiogenesis in

| Table 4 Correlation between the microvessel density 1 in relation to the grade of cancer |
|-----------------|------------------|------------------|------------------|
| MVD1           | Range            | Mean ± SD        | ANOVA F         | P-value         |
| I              | 13.25–51.20      | 29.988 ± 11.335  | 3.212           | 0.05*           |
| II             | 22.00–83.20      | 37.472 ± 14.205  |                |                 |
| III            | 32.80–87.20      | 47.100 ± 20.004  |                |                 |
| Total          | 13.25–87.20      | 36.686 ± 15.357  |                |                 |

ANOVA, analysis of variance; MVD, microvessel density; *Statistically significant.

| Table 5 The difference between initial microvessel density for nonrecurrent cases and microvessel density in cases that developed recurrence |
|-------------------------------------------------------------|-----------------|-----------------|-----------------|
| MVD1            | Range            | Mean ± SD        | Mann–Whitney U-test |
| MVD of nonrecurrent case (n = 32)                          | 17.2–49         | 32.972 ± 7.912  | –1.522          | 0.128 |
| MVD of recurrent cases (n = 8)                             | 13.25–87.2      | 49.956 ± 26.91  |                 |      |

MVD, microvessel density.
cancers, the determination of MVD (quantification of microvessels stained with immunohistochemical stains in histological sections) is considered to be a reliable indicator [16]. Yet, the measurement of microvasculature in histological sections does not necessarily represent the angiogenic status of the tumour [30,31]. Another reason for these conflicting results is that the sections analysed may not represent the true state of tumour angiogenesis.

The standardization of antibodies and immunohistochemical techniques and the use of automated methods for the determination of vascularity could eliminate some of these problems, but the use of these techniques requires highly specialized equipment [32]. The use of a computer-assisted analysis of the microvasculature in tumours seems to be promising, but the wide use of these techniques requires special training and equipment [33]. It is obvious that the debate for the ideal method for microvessel counting still exists. Moreover, antibodies used to highlight blood vessels cannot distinguish between resting and active angiogenic vessels.

In addition, there is no agreement about which types of antibodies should be used in microvessel counting, with different researchers using different antibodies [8,31,33], which may contribute to varied results. Moreover, some studies include patients treated with different therapeutic modalities (surgery or radiation), which can be influenced in different ways by tumour vascularization [15].

### Conclusion

Our results demonstrate that tumours associated with a higher mean microvessel count are associated with a more aggressive nature, such as those with nodal metastasis, or recurrence, and tumours arising from sites with poorer prognosis. This relation was also observed with tumours with a higher T stage, although it was not statistically significant. These results suggest that angiogenic activity can be used as a prognostic factor in patients with LSSC; further, it can direct laryngologists to a more aggressive approach in the treatment and follow-up of such tumours.

### Table 6 Comparison between microvessel density 1 and microvessel density 2 in recurrent cases

<table>
<thead>
<tr>
<th>MVD</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>Wilcoxon signed-rank test</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVD1</td>
<td>13.25–87.2</td>
<td>49.956 ± 26.91</td>
<td>−2.38</td>
</tr>
<tr>
<td>MVD2</td>
<td>37–100.8</td>
<td>72.525 ± 22.344</td>
<td></td>
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MVD, microvessel density; *Statistically significant.

### Acknowledgements

None declared.

### References

19. MVD, microvessel density; *Statistically significant.


