学位論文

「Microenvironmental changes in the progression from adenocarcinoma in situ to minimally invasive adenocarcinoma and invasive lepidic predominant adenocarcinoma of the lung」（置換型肺腺癌における上皮内腺癌から微少浸潤癌、浸潤型置換型肺腺癌への進展過程における微小環境の変化）

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著者の宣言

本学位論文は、著者の責任において実験を遂行し、得られた真実の結果に基づいて正確に作成したものに相違ないことをここに宣言する。

[要旨]

【目的】

近年 WHO 分類が改訂され、新たに置換型肺腺癌の分類が提唱された。すなわち adenocarcinoma in situ (AIS) 、minimally invasive adenocarcinoma (MIA)、invasive lepidic predominant adenocarcinoma (LPA)である。これら置換型肺腺癌である AIS、MIA、LPA は多段階に進行していくと考えられているが、このメカニズムについてはまだ解明されていないことが多い。

一方で、肺腺癌では腫瘍微小環境が腫瘍の悪性度に関わっているいるという報告はあるものの、置換型肺腺癌との関連については研究されていない。置換型肺腺癌の多段階発達に微小環境が関与している可能性は高いと考え本研究をすることとした。

【対象と方法】

我々はまず対象患者として 2003 年 1 月から 2010 年 3 月までに国立がんセンター東病院で肺癌に対して手術を受けた 1299 名から置換型肺腺癌の患者 270 名を抽出した。次に抽出した患者 270 名の組織検体に HE 染色行い、WHO 分類（第 4 版）に基づいて病理組織学的に 4 グループに分類した。4 グループは AIS (n=51)、MIA (n=59)、LPA-S (LPA かつ腫瘍径が 3 cm 以下、n=113)、LPA-L (LPA かつ腫瘍径が 3 cm より大きい、n=47)とした。4 グループに対して臨床病理学的な検討を行った。さらに病理学的検討を行うために、我々は AIS、MIA、LPA-S、LPA-L である症例を 2011 年 1 月から 2014 年 3 月にまでに国立がんセンター東病院で置換型肺腺癌に対して手術を受けた患者より各 20 例（合計 80 例）ずつ抽出した。これらの検体に対して種々の免疫染色を行いそれぞれ 4 グループの差を比較し、それぞれの腫瘍細胞の特徴および腫瘍微小環境の変化を研究することとした。

免疫染色は非浸潤部では腫瘍細胞以外の腫瘍微小環境因子をほぼ認めないため、浸潤部と非浸潤部（病理学的に lepidic type の形態を呈する腫瘍細胞を有する部位）でそれぞれ評価を行うこととした。免疫染色マーカーとしては EMT 関連マーカー(E-cadherin、S100A4)、浸潤マーカー(laminin-5、ezrin)、幹細胞関連マーカー(ALDH-1)、成長因子受容体マーカー(c-Met、 EGFR)の評価を各 20 症例に対して非浸潤部、浸潤部の腫瘍細胞でそれぞれ行った。微小環境因子としては Podoplanin-positive cancer-associated fibroblasts (PDPN+ CAFs)、 CD204-positive tumor-associated macrophages (CD204+ TAMs)と CD34+ microvessel cells の評価を浸潤部で行った。

【結果】

臨床的病理学的な統計結果では MIA と比較し LPA-S で血管浸潤および胸膜浸潤が有意に高かったが、LPA-S と LPA-L で有意な差は認められなかった。しかしながら、5 年無再発生存率を見ると LPA-L は他のグループと比較し有意に低い($p<0.01$)という結果であった。

免疫染色の結果では、非浸潤部での Laminin-5 の発現が MIA で AIS と比較して有意に高かった($p<0.001$)。MIA から LPA-S への発達段階では浸潤部での Laminin-5 の発現が MIA から LPA-S へ発達する段階で有意に高かった($p<0.001$)。さらに腫瘍微小環境因子として、LPA-S の浸潤部では PDPN+ CAFs、CD204+ TAMs、CD34+ microvessel が有意に高くなっていた（それぞれ $p<0.05$、$p<0.001$、$p<0.05$)。また 浸潤部での Ezrin の発現が LPA-S と比較して LPA-L で有意に高くなっていた($p<0.05$) 。しかしながら、浸潤部の腫瘍微小環境因子は LPA-S から LPA-L へ発達する段階で 有意な変化を認めなかった。

【考察】

本研究での免疫染色結果では MIA から LPA-S へ進行する際に最も変化が起きていた。浸潤マーカーである Laminin-5 の発現は特に浸潤部腫瘍細胞で上昇しており、この発達段階で腫瘍の浸潤度が上昇していくことが示唆された。さらに、腫瘍微小環境因子として PDPN+ CAFs、CD204+ TAMs、CD34+ microvessel の発現レベルが上昇していた。これらの腫瘍微小環境マーカーの発現上昇は、腫瘍悪性度と相関することが今まで報告されてきており、このことから、LPA-S の段階で腫瘍浸潤部では腫瘍細胞の浸潤能が増加し、さらに腫瘍周囲微小環境が腫瘍の悪性度を高めていると考えられる。一方でこれら微小環境因子は LPA-S から LPA-L および AIS から MIA への腫瘍発達段階では発現レベルが上昇していなかった。この現象は、腫瘍細胞の浸潤能増加と、悪性度を高める微小環境の変化が AIS から LPA に進行する際に重要な役割を担っている可能性を示唆する結果である。

MIA の非浸潤部と言われる部位で、浸潤マーカーである Laminin-5 が上昇していることも本研究では特徴ある結果である。過去の報告では Mathieu らが matrix metalloproteinases (MMPs)が肺腺癌の進行に関与していることを報告し、さらに腫瘍進行度と相関関係がある MMP-13 の発現が AIS と比較し、MIA で上昇していたと報告している。この報告は我々の今回の研究結果と矛盾していない。また Kanomata らは MMPs の肺腺癌における発現レベルが非浸潤部と浸潤部でほぼ変わらないと報告している。これらのことと我々の今回の研究結果を合わせて考えると、MIA の段階ですでに非浸潤部と言われる部位も浸潤能を有する可能性が示唆される。

【結語】

本研究では AIS から肺腺癌がどのように発達していくかのメカニズムを置換型肺腺癌の形態的特徴である非浸潤部と浸潤部に分けて、免疫染色を行うことで研究した。研究結果では AIS から MIA へ腫瘍が発達する段階で、非浸潤部と今まで呼ばれていた部位で腫瘍細胞が浸潤能を獲得し、次のステージである MIA から LPA-S へ腫瘍が発達する段階の浸潤部で腫瘍細胞の浸潤能の上昇と腫瘍周囲の悪性度を高める腫瘍微小環境の形成が起きていた。この腫瘍発達段階でおこる現象は置換型肺腺癌が AIS から LPA に進行していく過程で重要な役割を果たしている可能があると考えられる。

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1. Introduction

Small peripheral lung adenocarcinomas were previously classified by focusing on the morphological features. Noguchi et al classified small peripheral lung adenocarcinomas, which have bronchioalveolar components, into three types: Type A, localized bronchioloalveolar carcinoma; Type B, localized bronchioloalveolar carcinoma with foci of alveolar structural collapse; and Type C, localized bronchioloalveolar carcinoma with foci of active fibroblastic proliferation.(1) This classification is closely associated with clinical and radiological findings and would therefore reflect the process of development of lung adenocarcinoma. The current World Health Organization (WHO) classification (4^{th} edition) proposed the new categories for lung adenocarcinomas, such as adenocarcinoma in situ (AIS) and minimally invasive adenocarcinoma (MIA), on the basis of Noguchi's classification. (2, 3) AIS is defined as a tumor that grows in a lepidic fashion along pre-existing airway structures without detectable invasion. In some AIS cases, solid attenuation increases with the passage of time on thin slice CT; these cases show excellent prognosis (4-9) and AIS is considered to be a pre-invasive malignant lesion (carcinoma in situ). (3, 10) After the accumulation of further additional genetic/epigenetic abnormalities, AIS eventually generates an invasive component.

MIA is the next stage of progression of AIS and is defined as a small, solitary adenocarcinoma ($\leq$ 3 cm), with a predominantly lepidic pattern and $\leq$ 5 mm invasion in the greatest dimension. Furthermore, when the invasive area of MIA enlarges by more than 5 mm in diameter or meets invasion criteria, the lesion is defined as an invasive lepidic predominant adenocarcinoma (LPA). (2) A multistep carcinogenesis hypothesis suggests progression from AIS to MIA to LPA, but the underlying mechanisms of this progression are not fully understood.

The characteristics of the invasive component are key histological findings for determining the malignant potential of cells.(11-13) This component is composed of not only cancer cells but also several kinds of stromal cells, including fibroblasts, macrophages, inflammatory cells, and newly formed microvessels. These stromal cells can influence the proliferation, survival, and invasion of cancer cells and create a specific tumor microenvironment. (14-19) Therefore, to elucidate the stepwise progression mechanisms of cancer, it is necessary to evaluate the molecular characteristics of both cancer cells and stromal cells.

The kind of molecular expression changes that would occur in cancer cells in the process of development from AIS to LPA and the kind of stromal cells that are recruited are unclear. Investigating the relevant molecular expression levels during each stepwise may reveal the exact progression mechanism of LPA. In the current study, we

examined the immunohistological expression levels of cancer cells and stromal cells during each step and evaluated microenvironmental changes during the progression from AIS to LPA.

2. Materials and Methods

2.1. Patient selection

A total of 1299 patients underwent complete surgical resection for lung adenocarcinoma between January 2003 and March 2010 at National Cancer Center Hospital East, Chiba, Japan. Among these patients, 315 were diagnosed with LPA; 45 were excluded because of mucinous adenocarcinoma and multiple primary lung cancer. The remaining 270 cases were included in this study. According to the WHO classification of cell types, 4th edition, we divided the 270 cases into 4 groups: AIS (n=51), MIA (n=59), LPA-S (invasive adenocarcinoma smaller than 3 cm, n=113), and LPA-L (invasive adenocarcinoma lager than 3 cm, n=47). All the surgical specimen were collected and analyzed after receiving the approval of the Institutional Review Board of the National Cancer Center Hospital East.

2.2. Histological studies

The surgical specimens were fixed in 10 % formalin, embedded in paraffin, and serially sectioned at 4-mm intervals. The sections were stained using the hematoxylin and eosin (HE) method. The Alcian blue-periodic acid-Schiff (AB-PAS) method for the detection of cytoplasmic mucin production. All the histological materials included in this series were reviewed by two pathologists (M.N. and G.I.). The pathological stage was determined based on the TNM classification of the Union for International Cancer control (UICC), 7th edition. Each component (lepidic, papillary, acinar, solid, and micropapillary) was recorded as the percentage of the total tumor composition in 10% increments. The subtype present in the greatest proportion was defined as the predominant pattern. We classified lepidic predominant adenocarcinoma into three subtypes according to the WHO classification of cell types, 4th edition: (1) AIS, (2) MIA, and (3) LPA. AIS is defined as a $\leqq$3 cm tumor with only a lepidic component (Fig.1A, B). MIA is defined as a $\leqq$3 cm tumor with a lepidic predominant pattern and a $\leqq$ 5 mm invasive component (Fig.1C, D). LPA is defined as a > 3 cm tumor with a lepidic predominant pattern and/or a > 5 mm invasive component. Furthermore, we classified LPA into 2 groups: lepidic predominant invasive adenocarcinoma < 3 cm (LPA-S; Fig.1E, F) and lepidic predominant invasive adenocarcinoma > 3 cm (LPA-L: Fig.1G, H).

2.3. Antibodies and immunohistochemical staining

We selected 20 cases from each of the 4 groups (AIS, MIA, LPA-S and LPA-L) among complete surgical resection cases between January 2011 and March 2014. The antibodies used in this study were summarized in supplementary Table1. The slides were deparaffinized in xylene and dehydrated in a graded ethanol series, and endogenous peroxidase was blocked with 3 % hydrogen peroxide in absolute methyl alcohol. After epitope retrieval, the slides were washed with phosphate-buffered saline (PDL-1 was washed with TBST) and were incubated overnight at 4 °C using primary antibodies at their final dilution in the blocking buffer. The slides were washed again and incubated with EnVision (Dako, Glostrup, Denmark) for 1 h at room temperature. The reaction products were stained with diaminobenzidine; lastly, the slides were counterstained with Meyer hematoxylin.

2.4. Immunohistochemical scoring

All the stained tissue sections were semiquantitatively scored and evaluated independently under a light microscope by two pathologists (M.N. and G.I.). Because the characteristics of invasive component is predicted as key histological findings to determine the malignant potential, we evaluated staining score of tissue sections separating non-invasive component and invasive component. The area of non-invasive component and invasive component were also evaluated by two pathologist (M.N. and G.I.). The immunostaining scores of E-cadherin, S100A4, laminin-5, Ezrin, C-met, ALDH1, EGFR, were evaluated based on the staining intensity and the percentage of cancer cells. The following scoring system was used: 0 (negative staining, defined as no immunoreactivity); 1+ (weak staining intensity); and 2+ (strong staining intensity). We also evaluated the extent of staining in a lesion corresponding to every ten percentages (0–100 %). The staining scores were calculated by multiplying the percentage values by the staining intensity, with the scores ranging from 0 to 200. The number of CD204+ TAMs in stroma and CD34-positive microvessels cells were counted in three high-power microscopic fields in invasive area, and the averages were determined. Scoring of PDPN+ CAFs was evaluated based on the staining intensity and the percentage of area by above mentioned method.

2.5. Statistical analysis

Differences in categorical outcomes were evaluated using the chi-square test. The length of the recurrence-free survival was calculated in days from the date of resection until the date of the first recurrence or last follow up. Recurrence-free survival were calculated using the Kaplan-Meier method, and difference between the groups were

analyzed using a log-rank test. All the P values reported were 2-sided, and the significance level was set at <0.05. Differences in immunohistochemical characteristics between each groups (AIS, MIA, LPA-S, LPA-L) were calculated by using the Wilcoxon signed-rank test. Analyses was performed using the statistical software JMP10.

3. Results

3.1. Clinicopathological factors for AIS, MIA, LPA-S, and LPA-L

The clinicopathological factors for all 270 patients were shown in Table 1. Vascular invasion and pleural invasion were significantly higher in LPA-S than in MIA. However, no significant associations were found between LPA-S and LPA-L in relation with vascular invasion and pleural invasion.

Supplementary Fig. 1 showed the recurrence-free survival curve for the different groups. The 5-year RFS rates of AIS, MIA, LPA-S, and LPA-L were 100, 100, 93, and 73% respectively. The 5-year RFS of LPA-L was significantly lower than that of other groups (all $P < 0.01$).

3.2. Comparison of immunostaining scores of cancer cells in the non-invasive component of AIS, MIA, LPA-S, and LPA-L

EMT –related molecules:

There was no significant difference in the expression levels of E-cadherin and S100A4 between each group (Fig.2A, supplementary Table 2).

Invasion related molecules:

The average staining scores (and ranges) for laminin-5 in the noninvasive component in each group (AIS, MIA, LPA-S, and LPA-L) of cancer cells were 0 (0), 3.5 (0-20), 6.0 (0-50), and 4.0 (0-20), respectively. Fig. 2A, B, and supplementary Table 2 showed the expression level of laminin-5 in the noninvasive component of MIA was significantly higher than that in AIS ($P < 0.01$). There were no significant differences in the ezrin expression level in the noninvasive components for the groups (Fig.2A, supplementary Table 2).

Growth factor receptor:

No significant differences were observed between the expression levels of C-met or EGFR in any of the groups (Fig.2A, supplementary Table 2).

Stem-cell related molecule:

There were no significant differences between the expression levels of ALDH1 in any of the groups (Fig.2A, supplementary Table 2).

3.3. Comparison of immunostaining scores of cancer cells in the invasive component of MIA, LPA-S, and LPA-L

EMT-related molecules:

There were no significant differences between expression levels of E-cadherin or S100A4 in any of the groups (Fig.3A, supplementary Table 3).

Invasion related molecules:

The average staining scores (and ranges) for laminin-5 were 4.5 (0-40), 21.5 (0-80), and 28.0 (0-160) for MIA, LPA-S, and LPA-L respectively. The average staining scores (ranges) for ezrin were 27.0 (0-90), 16.5 (0-60), and 44.0 (0-80) for MIA, LPA-S, and LPA-L, respectively. Fig.3A, B, and supplementary Table 3 showed the expression level of laminin-5 in the invasive component of LPA-S was significantly higher than that in MIA ($P < 0.01$). Fig.3A, B, and supplementary Table 3 showed the expression level of ezrin in the invasive component of LPA-L was significantly higher than that in LPA-S ($P = 0.03$).

Growth factor receptor:

There were no significant differences between the expression levels of C-met or EGFR in any of the groups (Fig.3A, supplementary Table 3).

Stem-cell related molecule:

There were no significant differences between expression levels of ALDH1 in any of the groups (Fig.3A, supplementary Table 3).

3.4. Comparison of immunostaining scores of stromal cells in the invasive component of MIA, LPA-S and LPA-L

M2 macrophage:

The average numbers (and ranges) of CD204+ TAMs in each group were 0.05 (0-1), 4.2 (0-14), and 7.2 (0-24) for MIA, LPA-S, and LPA-L, respectively. Fig.4A, B, and supplementary Table 3 showed the densities of CD204+ TAMs in the stroma of LPA-S were significantly higher than those of MIA ($P < 0.01$).

CD34+ microvessel:

The average numbers (and ranges) of CD34+ microvessels in the stroma of each group were 4.8 (0-19), 8.6 (2-19), and 10.8 (3-19) for MIA, LPA-S, and LPA-L, respectively. Fig.4A, B, and supplementary Table 3 showed the densities of CD34+ microvessels in the stroma of LPA-S were significantly higher than those in MIA ($P < 0.01$).

Podoplanin-positive CAFs:

The average staining scores (ranges) for PDPN+ CAFs in each group were 0 (0), 5 (0-30), and 3.5 (0-40) for AIS, MIA, LPA-S, and LPA-L, respectively. Fig.4A, B, and supplementary Table 3 showed the expression level of PDPN+ CAFs in the invasive

component of LPA-S was significantly higher than that in MIA ($P = 0.02$).

4. Discussion

Lung adenocarcinoma progression has been inferred from morphological, radiological, and molecular findings. Noguchi et al. classified small peripheral lung adenocarcinoma, focusing on the morphological features of the invasive component (17). Based on these findings, the current WHO classification (4th edition) proposed the categories of AIS, MIA, and LPA as stepwise progressions of lepidic predominant adenocarcinoma. However, little study have reported this stepwise morphological progression with regard to the molecular expression of cancer cells and stromal cells. The present study is the first to address this problem.

In this study, the most drastic changes in molecular expression were observed during the progression from MIA to LPA-S. Recent immunohistochemical studies have revealed that the expression level of laminin-5 and invasion-related molecules (20-22) in the invasive carcinoma component increased drastically during the progression from MIA to LPA-S. These results indicated that the invasiveness of cancer cells may change at the initial invasive stage. Remarkably, the numbers of tumor-promoting stromal cells including PDPN+ CAFs, CD204+ TAMs, and CD34+ microvessels were increased during the progression from MIA to LPA-S. Since these stromal cells are reported to be required for further tumor progression (23-27), increased numbers of both laminin-5-expressing cancer cells and tumor-promoting stromal cells would lead to the formation of a more tumor-promoting microenvironment in LPA-S. On the other hand, these tumor-promoting stromal cells did not increase during progression of LPA-S to LPA-L, or of AIS to MIA. It is reasonable to think that these microenvironmental changes, caused by cancer cells with an invasive phenotype and stromal cells with a tumor-promoting phenotype, might play an important role in the disease progression from AIS to LPA.

Mathieu et al examined the expression of matrix metalloproteinases (MMPs) throughout lung adenocarcinoma development (28). They identified MMP-13 expression as a potential marker of progression of lung adenocarcinoma, as expression levels of MMP-13 were significantly increased in MIA compared to AIS. Consistent with these findings, our study also identified that the noninvasive component of MIA showed significantly higher expression of the invasion-related molecule laminin-5, compared to AIS ($P < 0.01$). Kanomata et al investigated mRNA expression of MMP2, MMP9, MT1-MMP, and TIMP2 in mixed-type lung adenocarcinomas in noninvasive and invasive areas using in situ hybridization (7). They found that the expression levels of

the MMPs in the noninvasive component were similar to those in the invasive component. They also showed that fibroblasts located in the thickened alveolar septa in the non-invasive component were the source of MMP-2, MMP-9, and TIMP2, and that cancer cells and stromal cells exhibited similar levels of MT1-MMP mRNA in both the invasive and noninvasive components. Considering these findings, our results suggest that the lepidic component (noninvasive component) already has invasive potential at the stage of MIA.

Our study indicated that patients with LPA-S displayed shorter RFS than the patients with LPA-L ($P < 0.01$). During progression from LPA-S to LPA-L, the expression of invasion related molecules such as ezrin was significantly increased in the invasive component of cancer cells ($P= 0.03$). Ezrin expression in lung cancer is reported to be correlated with local invasion (29, 30), lymphatic metastasis, and advanced TNM stage (29, 31). Thus high expression levels of ezrin are attributed to the high frequency of lymphovascular invasion and poor prognosis of LPA-L. On the other hand, laminin-5 expression levels in the invasive components in LPA-L and LPA-S were similar in this study. It can be assumed that ezrin is a stronger marker of metastasis and prognosis of LPA than laminin-5.

5. Conclusion

Figure 5 shows a summary scheme of the stepwise progression of LPA based on the results of this study. During the progression from AIS to MIA, the invasive potential of cancer cells is already increased in the noninvasive component. At the progression from MIA to LPA-S, microenvironmental molecular changes occur, characterized by increased invasive phenotype of cancer cells and increased recruitment of tumor-promoting stromal cells. Furthermore, during the progression from LPA-S to LPA-L, the expression level of ezrin increased. Although the molecular mechanisms of the changes in these expression levels at each progression stage are not clear, these results might provide a better understanding of the mechanism of stepwise progression in LPA.

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7. Figure legends

Fig.1 Microscopic features of AIS, MIA, LPA-S, and LPA-L.
a. Low-power view of AIS. **b.** High-power view of AIS. **c.** Low-power view of MIA. **d.** High-power view of the invasive component of MIA. **e.** Low-power view of LPA-S. **f.** High-power view of the invasive component of LPA-S. **g.** Low-power view of LPA-L. **h.** High-power view of the invasive component of LPA-L.
Fig.2 Comparison of immunohistochemical staining scores of cancer cells in the non-invasive component. **A.** The expression levels of E-cadherin, S100A4, laminin-5, ezrin, C-met, EGFR, and ALDH1. Each antibody in each group was examined using the Wilcoxon signed-rank test. **B.** Comparison of immunohistochemical staining of laminin-5 in the noninvasive component at AIS and MIA.
Fig.3 Comparison of immunohistochemical staining scores of cancer cells in the invasive component. **A.** The expression levels of E-cadherin, S100A4, laminin-5, ezrin, C-met, EGFR, and ALDH1. Each antibody in each group was examined using the Wilcoxon signed-rank test. **B.** Comparison of immunohistochemical staining of laminin-5 in the invasive component at MIA and LPA-S, and ezrin in the invasive component at LPA-S and LPA-L.

Fig.4 Comparison of immunohistochemical staining scores of stromal cells in the invasive component. **A.** The expression levels of CD204+ TAMs, CD34+ microvessels, and PDPN+ CAFs in the invasive component. Each antibody in each group was examined using the Wilcoxon signed-rank test. **B.** Comparison of immunohistochemical staining of CD204+ TAMs, CD34+ microvessels, and PDPN+ CAFs in the invasive component at MIA and LPA-S.
Fig.5 Scheme of stepwise progression from AIS to LPA-L. During the progression from AIS to MIA, the expression level of laminin-5 increased. During the progression from MIA to LPA-S, the expression of the invasion-related marker (laminin-5) in cancer cells was elevated. Moreover, the number of PDPN+ CAFs, CD204+ TAMs, and CD34+ microvessels of stromal cells in the invasive component also increased. Furthermore, during the progression from LPA-S to LPL-L, invasion related marker expression (ezrin) increased in LPL-L.

Figure 1

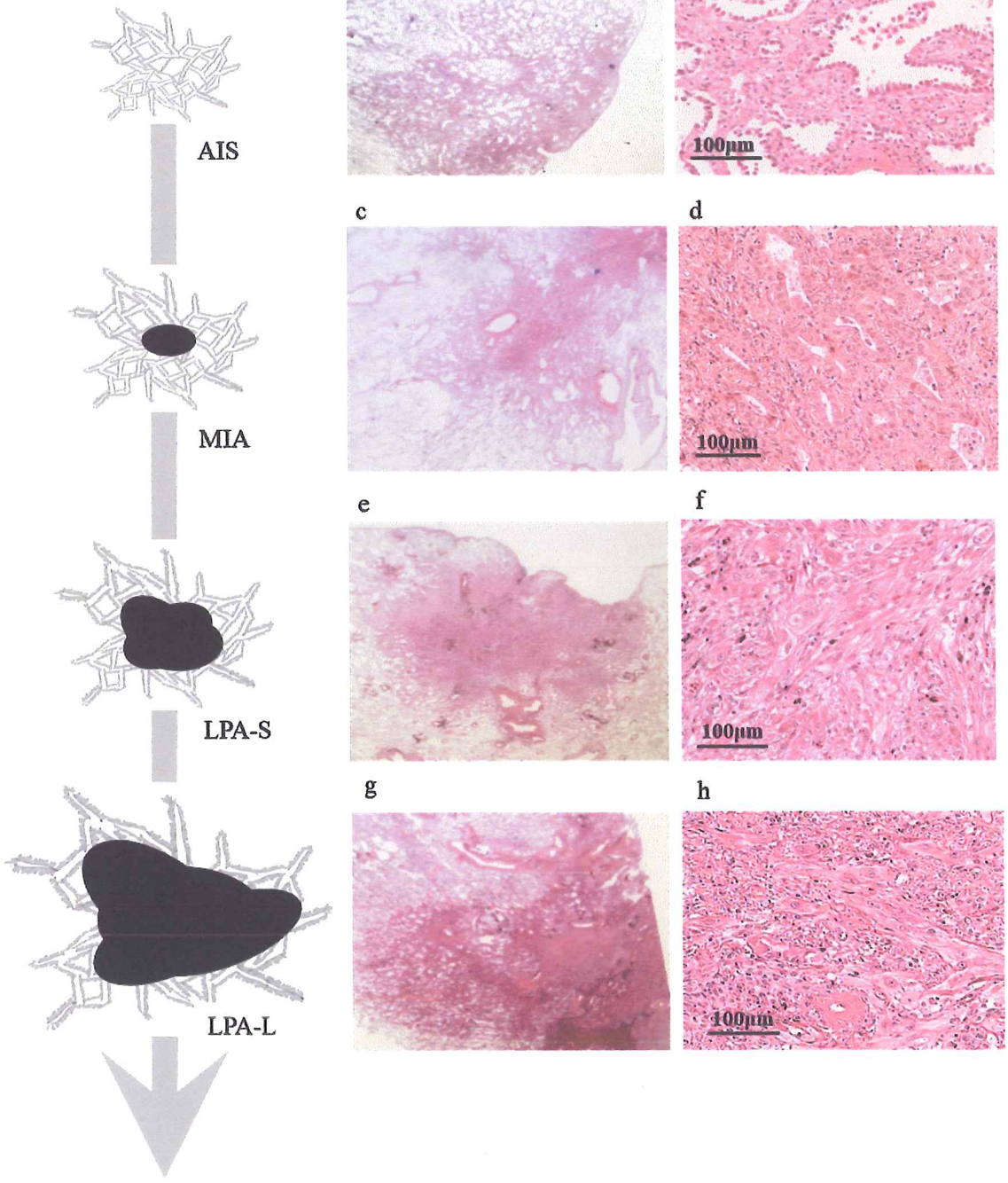
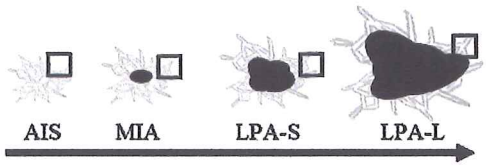
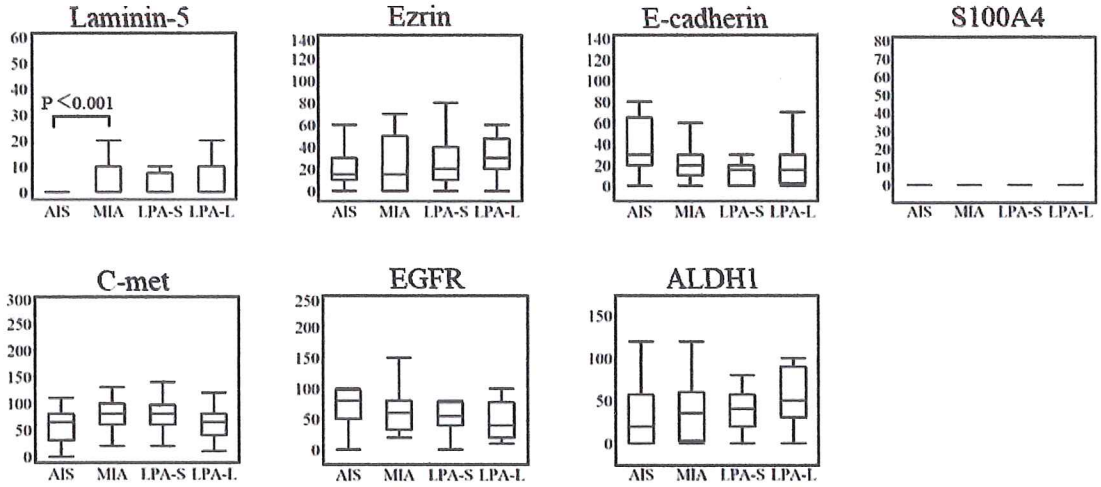


Figure 2


AIS
MIA
LPA-S
LPA-L

A


Laminin-5
P<0.001
Ezrin
E-cadherin
S100A4
C-met
EGFR
ALDH1
AIS MIA LPA-S LPA-L

B

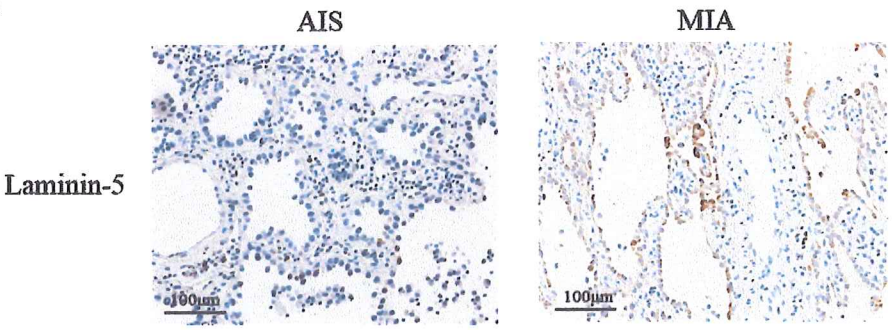

AIS
MIA
Laminin-5
100μm
100μm

Figure 3

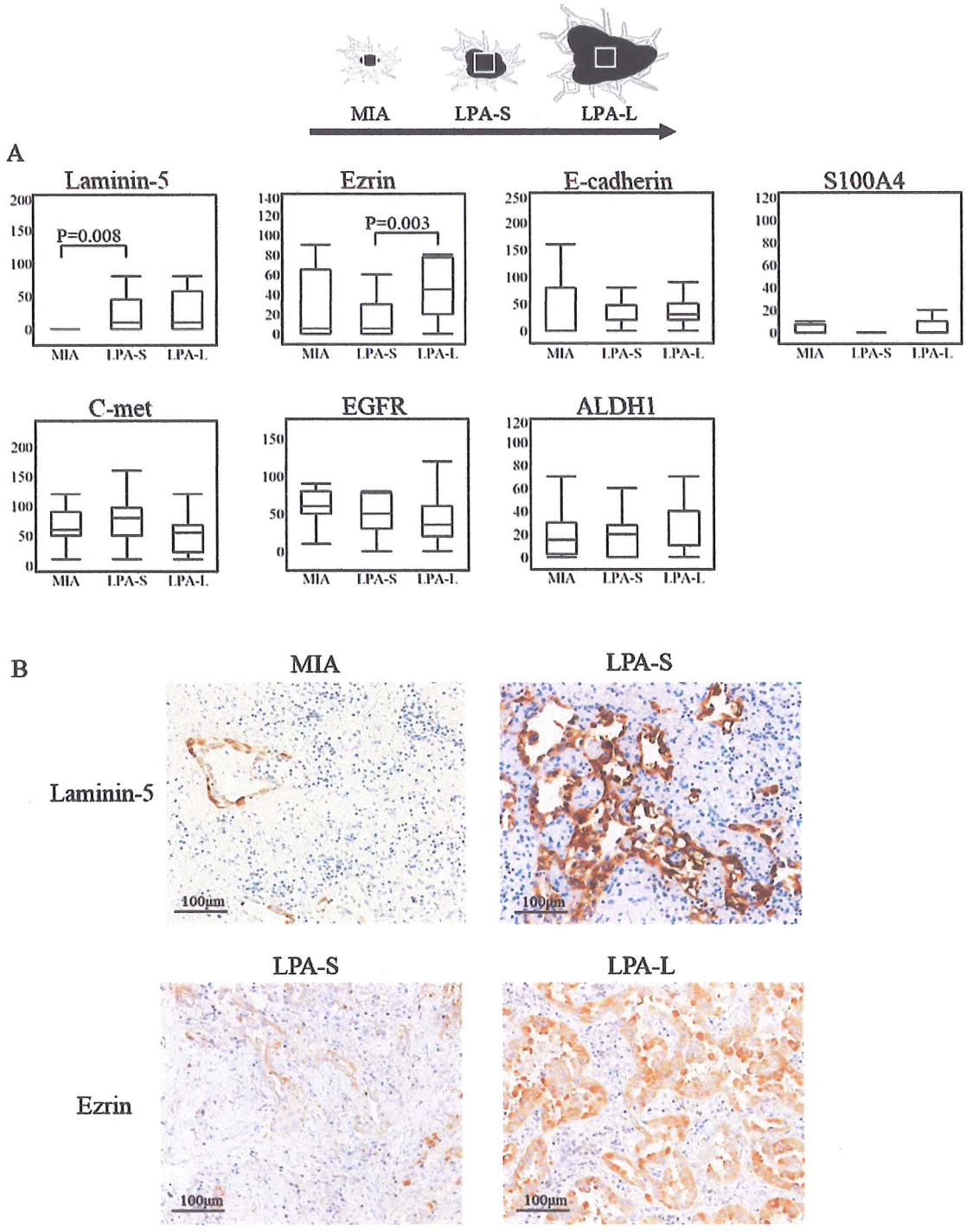
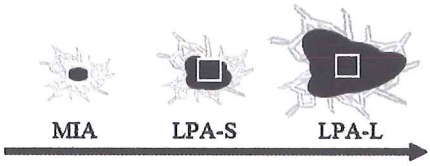
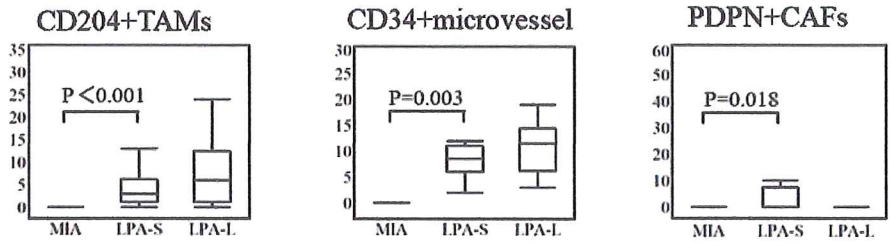

MIA
LPA-S
LPA-L
A
Laminin-5
P=0.008
Ezrin
P=0.003
E-cadherin
S100A4
C-met
EGFR
ALDH1
MIA
LPA-S
LPA-L
B
MIA
LPA-S
Laminin-5
100μm
LPA-S
LPA-L
Ezrin
100μm

Figure 4


MIA
LPA-S
LPA-L

A


CD204+TAMs
P <0.001
CD34+microvessel
P=0.003
PDPN+CAFs
P=0.018
MIA
LPA-S
LPA-L

B

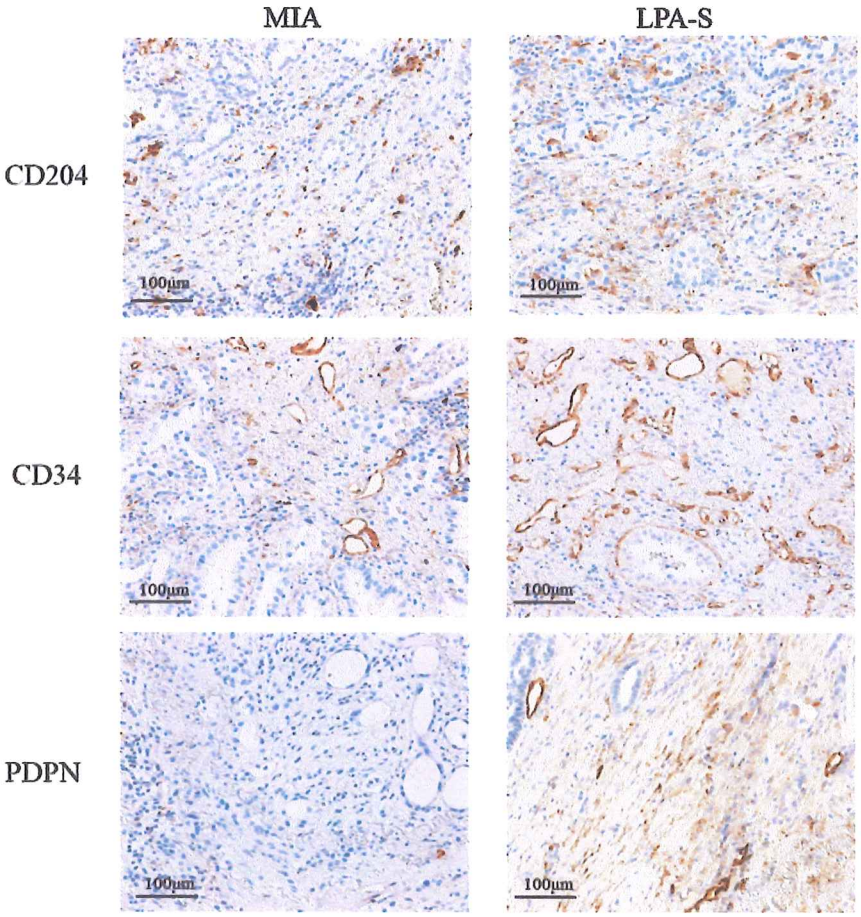

MIA
LPA-S
CD204
CD34
PDPN
100μm

Figure 5

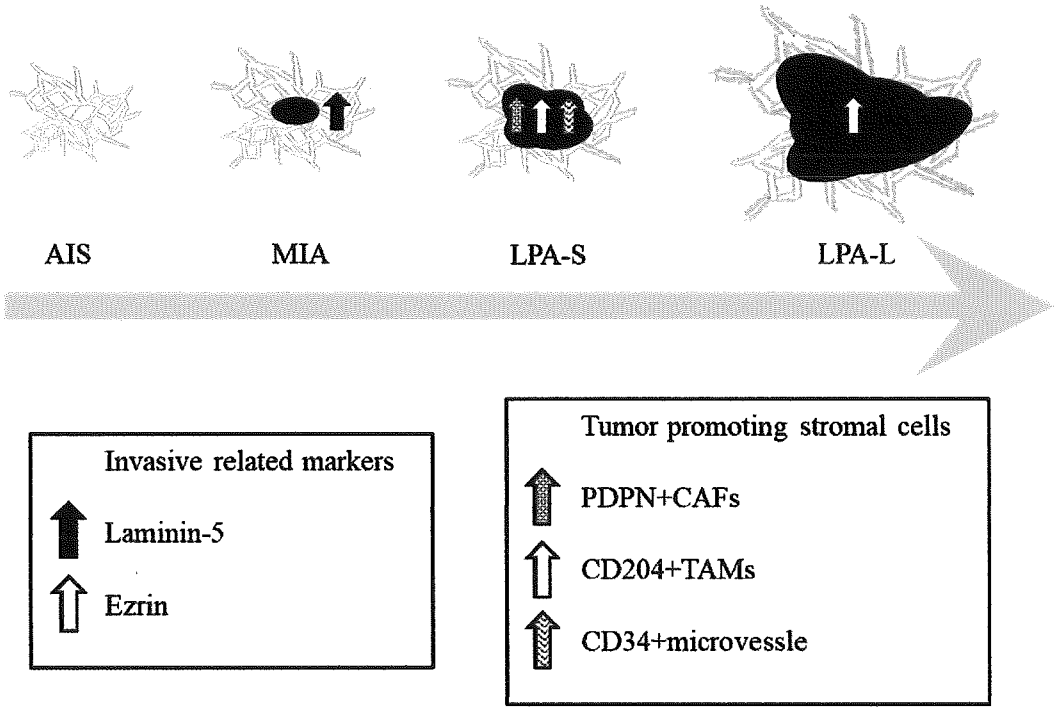
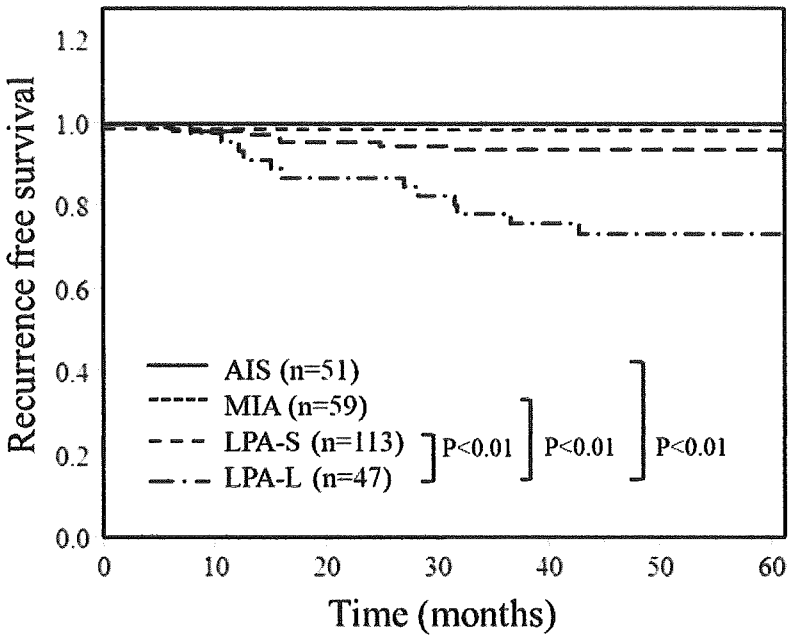

AIS
MIA
LPA-S
LPA-L
Invasive related markers
Laminin-5
Ezrin
Tumor promoting stromal cells
PDPN+CAFs
CD204+TAMs
CD34+microvessle

Table. 1 Clinicopathological factors of AIS, MIA, LPA-S and LPA-L

variables	AIS	*P	MIA	**P	LPA-S	***P	LPA-L
No. of patients	51		59		113		47
Age(median(range))	63 (41-78)	0.60	64 (35-84)	0.09	67 (37-83)	0.25	69 (51-84)
Sex		0.06		0.43		0.31	
Male	13		25		55		27
Female	38		34		58		20
Smoking		0.81		0.17		0.77	
Never	27		36		59		26
Ever	24		23		54		21
Surgical resection		0.02		< 0.01		0.19	
Lobar	34		50		109		47
Sublobar	17		9		4		0
Vascular invasion		-		0.01		0.57	
Absent	51		59		102		41
Present	0		0		11		6
Lymphatic invasion		-		0.10		0.30	
Absent	51		59		108		43
Present	0		0		5		4
Pleural invasion		-		0.01		0.25	
Absent	51		59		103		40
Present	0		0		10		7

*AIS vs MIA, ** MIA vs LPA-S, ***LPA-S vs LPA-L

Supplementary Figure 1



Supplementary Table 1 Antibodies used

Category	Antibody	Clone	Preparation	Source
EMT	E-cadherin	NCH-38	Microwave	Dako Cytomation, Captinteria, CA
	S100A4	6275	Microwave	Acris Antibodies, Inc
Cell Invasion	Lamominin-5	Proteinase K	Proteinase K	Millipore
	Ezrin	-	Microwave	Cell signaling Technology, Inc
Growth factor receptor	C-met	8F11	Microwave	Leica Microsysrems
	EGFR	EGFR.113	Microwave	Leica Microsysrems
Stem cell	ALDH1	44/ALDH	Microwave	BD Transduction Laboratories
M2 macropharge	CD204	SRA-E5	Microwave	Trans Genic Inc
microvessel	CD34	QBEND/10	Microwave	Acris Antibodies, Inc
CAFs	Podoplanin	D2-40	Microwave	SIGNET

Supplementary table 2
Immunophenotype of cancer cells in non-invasive carcinoma component of AIS, MIA, LPA-S and LPA-L

		AIS	MIA	LPA-S	LPA-L
EMT –related molecules					
E-cadherin	average	35.5	39.5	37.5	53.2
	(range)	(0-160)	(0-120)	(0-100)	(0-100)
	P-value	0.56	0.59	0.42	
S100A4	average	0	3.0	5.0	2.5
	(range)	(0)	(0-30)	(0-70)	(0-40)
	P-value	0.07	0.98	0.62	
Invasion related molecules					
Laminin-5	average	0	3.5	6.0	4.0
	(range)	(0)	(0-20)	(0-50)	(0-20)
	P-value	<0.01	1.00	0.94	
Ezrin	average	20	26.5	23.5	30.0
	(range)	(20-60)	(0-70)	(0-80)	(0-60)
	P-value	0.93	0.82	0.18	
Growth factor receptor					
C-met	average	55.0	80.5	78.5	65.5
	(range)	(0-110)	(20-180)	(20-140)	(10-120)
	P-value	0.09	0.83	0.17	
EGFR	average	67.5	60.5	56.5	46.0
	(range)	(0-100)	(20-150)	(0-160)	(10-100)
	P-value	0.19	0.71	0.32	
Stem-cell related molecule					
ALDH1	average	35.5	22.5	15.5	22.0
	(range)	(0-100)	(0-80)	(0-60)	(0-80)
	P-value	0.10	0.49	0.57	

Supplementary table 3
Immunophenotype of cancer cells in invasive carcinoma component of AIS, MIA, LPA-S and LPA-L

		MIA	LPA-S	LPA-L
EMT –related molecules				
E-cadherin	average	41.0	32.0	37.0
	(range)	(0-100)	(0-100)	(0-100)
	P-value	0.62	0.32	
S100A4	average	7.5	5.5	3.5
	(range)	(0-30)	(0-70)	(0-40)
	P-value	0.46	0.36	
Invasion related molecules				
Laminin-5	average	4.5	21.5	28.0
	(range)	(0-40)	(0-80)	(0-160)
	P-value	< 0.01	0.94	
Ezrin	average	27.0	16.5	44.0
	(range)	(0-90)	(0-60)	(0-80)
	P-value	0.16	< 0.01	
Growth factor receptor				
C-met	average	67.4	75.0	53.5
	(range)	(10-120)	(10-160)	(10-120)
	P-value	0.55	0.07	
EGFR	average	64.0	51.5	41.5
	(range)	(10-150)	(0-160)	(0-120)
	P-value	0.12	0.28	
Stem-cell related molecule				
ALDH1	average	20.0	20.0	23.5
	(range)	(0-70)	(0-70)	(0-70)
	P-value	0.72	0.62	
M2 macrophage				
CD204	average	0.05	4.2	7.2
	(range)	(0-1)	(0-14)	(0-24)
	P-value	< 0.01	0.14	
CD34-positive microvessel				
CD34	average	4.8	8.6	10.8
	(range)	(0-19)	(2-19)	(3-19)
	P-value	< 0.01	0.11	
Podoplanin-positive CAFs				
Podoplanin	average	0	5	3.5
	(range)	(0)	(0-30)	(0-40)
	P-value	0.02	0.65	

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