Histol Histopathol (2016) 31: 1113-1122

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Mesotheliomas show higher hyaluronan positivity around tumor cells than metastatic pulmonary adenocarcinomas

Kari Törrönen[1], Ylermi Soini[2,3], Paavo Pääkkö[4], Jyrki Parkkinen[1], Reijo Sironen[2,3] and Kirsi Rilla[1]

[1]Institute of Biomedicine, University of Eastern Finland, Kuopio, [2]Institute of Clinical Medicine, Pathology and Forensic Medicine and Department of Pathology, Kuopio University Hospital, Kuopio, [3]Cancer Center of Eastern Finland, Kuopio and [4]Department of Pathology, Oulu University Hospital, Oulu, Finland

Summary. Hyaluronan is a unique glycosaminoglycan of the extracellular matrix, abundant in normal connective tissues but highly increased in many pathological conditions like cancer. Mesothelioma, one of the most malignant cancer types, is associated with high content of hyaluronan, with elevated levels of hyaluronan in pleural effusions and serum of the patients. Metastatic lung adenocarcinomas are typically less aggressive and have a better prognosis as compared to mesotheliomas, a reason why it is highly important to find reliable tools to differentiate these cancer types.

The main purpose of this study was to evaluate the amount of hyaluronan, hyaluronan producing synthases (HAS's) and hyaluronan receptor CD44, in mesothelioma and metastatic lung adenocarcinomas. Furthermore, we wanted to clarify the role of hyaluronan, CD44 and HAS's as putative markers for differentiating malignant mesothelioma from metastatic lung adenocarcinomas.

The main finding of this study was that mesotheliomas are significantly more positive for hyaluronan staining than metastatic adenocarcinomas. Unexceptionally, a trend of CD44 positivity of stromal cells was higher in adenocarcinomas as compared to mesotheliomas. However, no statistically significant differences were found between the staining of any of the HAS isoenzymes either in tumor cells or stromal cells of different groups of cases.

The results show that there are significant differences in hyaluronan content between metastatic lung adenocarcinomas and mesotheliomas. However, as previous studies have suggested, hyaluronan alone is not a sufficient independent marker for diagnostic differentiation of these cancer types, but could be utilized as a combination together with other specific markers.

Key words: Mesothelioma, Hyaluronan, CD44, Hyaluronan synthase

Offprint requests to: Kari Törrönen, Institute of Biomedicine, University of Eastern Finland, P.O.B. 1627, FIN-70211 Kuopio, Finland, e-mail: kari.torronen@uef.fi

DOI: 10.14670/HH-11-740

Introduction

Mesothelioma is an aggressive cancer type arising from the mesothelium, a membrane that covers and protects most of the body's internal organs. The two most common sites where mesothelioma develops are pleural and peritoneal mesothelia (Robinson and Lake 2005). Mesothelioma is a relatively rare cancer type. There are about 100 cases per year in Finland but the annual number of cases is increasing (Pukkala et al., 2009). Mesothelioma is an occupational disease and its prevalence is increased among shipyard and insulation workers. Exposure to asbestos is the main risk factor. Progress of the disease is very slow, taking normally decades after the exposure. The incidence of mesotheliomas is assumed to start to decrease in the western world after a couple of decades (Lee et al., 2007).

Mesothelioma is classified for epitheliod, sarcomatoid, and biphasic subtypes. All three subtypes

are very aggressive and the life expectancy of patients is extremely low (Robinson and Lake, 2005). A correlation between epithelial-to-mesenchymal markers and epitheliod to biphasic and to sarcomatoid histological subtypes has been shown recently (Fassina et al., 2012). However, the diagnosis of mesothelioma is still challenging. Lung adenocarcinoma and metastasis from some other cancers show a similar morphological picture as mesothelioma in routine histological stainings of lung biopsies. Novel immunohistochemical markers for diagnostics of epitheliod mesothelioma, like h-caldesmon, calretinin, estrogen receptor, Ber-EP4 f (Comin et al., 2007), D2-40 and podoplanin (Chirieac et al., 2011) have been developed.

Hyaluronan (HA) is a linear glycosaminoglycan, which is built up of repeating D-glucuronic acid and N-asetylglycosamine residues. The molecular mass of one hyaluronan molecule can reach up to millions of Daltons (Laurent and Fraser, 1992). Hyaluronan has a crucial role in embryonic development and morphogenesis (Li et al., 2007). Increased hyaluronan is associated with poor prognosis in many adenocarcinomas like breast, ovarian, colon or gastric cancers (Tammi et al., 2008). A hyaluronan-rich environment provides a favourable environment for initiation and progression of cancer, and high levels of hyaluronan in the tumor stroma or on the cancer cells coincides with an aggressive tumor type (Lipponen et al., 2001; Pirinen et al., 2001; Aaltomaa et al., 2002; Kosunen et al., 2004). However, the influence of hyaluronan is dependent on the size of the chain. Low molecular weight hyaluronan enhances angiogenesis and maintains inflammation (Slevin et al., 2004), while long hyaluronan chains prevent macrophage proliferation (Sheehan et al., 2004).

A family of three specific enzymes, hyaluronan synthases (HAS 1, HAS2, HAS3) are responsible for cellular production of hyaluronan. These enzymes are located at the inner face of the plasma membrane, synthesizing the hyaluronan chain directly to the extracellular space (Weigel et al., 1997). Each of the isoenzymes is capable of active hyaluronan production, but they have different enzymatic properties and functions (Itano et al., 1999). Expression of Has2 is essential during embryogenesis, while depletion of Has1 and Has3 show no special phenotype (Tien and Spicer 2005; Klewer et al., 2006).

Hyaluronan binding to its receptors, like CD44, RHAMM and LYVE activates intracellular signal transduction (Turley et al., 2002). The major hyaluronan binding molecule is CD44, which initiates various signalling events while binding hyaluronan (Ponta et al., 2003). CD44 exists in standard form and in several splice variant forms. It is also post-translationally modified. Different splicing forms of CD44 are increased in pathological conditions and are suggested to induce tumorigenicity (Naor et al., 2002; Misra et al., 2011).

Previous studies have shown that the levels of HAS1 and HAS2 are increased in mesotheliomas (Kanomata et al., 2005). Additionally, high hyaluronan content in pleural fluid correlates positively with short lifespan of patients (Thylen et al., 2001). It has been suggested that hyaluronan detection in lung tissue biopsy could act as a putative indicator of mesothelioma (Petersen et al., 2003).

In this study we investigated the expression of hyaluronan, hyaluronan synthases (HAS) and hyaluronan receptor CD44 in mesotheliomas and metastatic adenocarcinomas using paraffin embedded tissue sections. In addition, we wanted to find out if metastatic adenocarcinoma and mesothelioma could be differentiated by utilizing hyaluronan and hyaluronan-related proteins as diagnostic markers.

Materials and methods

Tumor samples

Archived formalin fixed, paraffin embedded tissue samples were collected at the Department of Pathology of Oulu University Hospital. The permits of ethical commission of Oulu University Hospital and National Authority for Medicolegal Affairs were obtained for this study. The material consisted of 39 mesotheliomas and 25 metastatic adenocarcinoma tissue samples. The mesothelioma samples consisted of 22 epithelioid, 12 sarcomatoid and 4 biphasic subtypes.

Hyaluronan staining

The descending xylene-ethanol series were used for rehydration of the sections. The endogenous peroxidases were blocked by incubating the sections with 3% H_2O_2 for 5 min. Unspecific binding of biotinylated hyaluronan binding complex (bHABC) was blocked by incubation with 1% bovine serum albumin (BSA) in 0.1 M Na-phosphate buffer, pH 7.0 (PB) for 30 min at 37°C. The sections were incubated overnight with 3 μg/ml bHABC prepared from bovine articular cartilage as described previously (Tammi et al., 1994). After washes with PB, the sections were incubated with avidin-biotin peroxidase (Vector Laboratories, Irvine, CA, 1:200) for 1 h. The color was developed with 0.05% 3,3'-diaminobenzidine (DAB, Sigma, St. Louis, MO) containing 0.03% H_2O_2. The sections were counter-stained with Mayer's hematoxylin for 2 min, washed, dehydrated, and mounted in DePex (BDH Laboratory Supplies, Poole, England). To control the specificity of the staining, hyaluronan was removed by preincubating the sections with *Streptomyces* hyaluronidase (Seikagaku, Kogyo, Tokyo, Japan), or the bHABC probe was blocked with HA-oligosaccharides (Tammi et al., 1994).

CD44 staining

Fixing and processing protocol of tissue sections was performed as described above. Endogenous

peroxidase activity and unspecific binding was blocked as described above. The sections were treated with anti-CD44 antibody (1:100, Hermes 3, a generous gift from Dr. Sirpa Jalkanen, Turku) overnight at 4°C. After washing, the sections were incubated with biotinylated anti-mouse secondary antibody (Vector Laboratories, 1:100) for 1 h at room temperature. Incubation with avidin-biotin peroxidase and color reaction with DAB was carried out as described above.

HAS stainings

The deparaffinized sections were treated for 5 min with 1% H_2O_2 to block endogenous peroxidase activity. Nonspecific binding was blocked by incubation of the sections in 1% BSA and 0.1% gelatine (Sigma G-2500, Sigma, MO) in PB for 30 min. Treatment of the sections with affinity purified polyclonal antibodies (2 μg/ml dilution in 1% BSA) for hyaluronan synthases HAS1 (sc-34021, Santa Cruz Biotechnology, inc., Santa Cruz, CA), HAS2 (sc-34067, Santa Cruz) or HAS3 (sc-34204, Santa Cruz), was carried out overnight at 4°C. After washes the samples were treated with biotinylated anti-goat antibody (1:1000, Vector Laboratories). The avidin-biotin peroxidase method was used to visualize primary-secondary antibody complexes. The primary antibodies were treated with corresponding peptides (Santa Cruz Biotechnology) to control the specificity of stainings. Mayer's hematoxylin was used as counterstain.

Evaluation of the stainings

The stainings were analyzed by two independent evaluators (KT and RS). The area of hyaluronan, HAS's, (HAS1, HAS2 and HAS3) and CD44 staining in the tumor cells and stroma was estimated with a five-level scoring from 0 to 4. Score 0 was given when no staining or staining on less than 5% of the area was detected. Score 1 was given, when 5-25% of the area was stained, score 2 when 26-50% of the area was stained, score 3 when 51-75% of the area was stained and score 4 when more than 76% of the area was stained. The intensity of staining was estimated with a four-level scoring from 0 to 3.

Statistical methods

The statistical analyses were performed using SPSS version 19.0 (IBM Corporation, Armonk, New York, USA). The differences between mesotheliomas and adenocarcinomas and the staining intensities of both stromal and tumor tissue for hyaluronan, CD44 and HAS1-3 were calculated with Fisher's exact test, which is suitable for a relatively small sample size. A difference was considered statistically significant when the p-value was less than 0.05. Correlations between different cancer and staining groups were tested with Pearson bivariate correlation. Test of significance was two-tailed.

Results

Mesotheliomas show higher hyaluronan positivity around tumor cells than metastatic adenocarcinomas

Images of hyaluronan stainings in epithelioid (Fig. 1a,d) and sarcomatoid (Fig. 1b,e) mesothelioma and adenocarcinoma (Fig. 1c,f) are shown in Fig. 1. Hyaluronan was found to localize both on the cell surfaces and in the extracellular space, but occasional intracellular staining was also detected. Most of the stromal tissue was positively stained for hyaluronan, and its intensity was typically moderate in epithelioid mesothelioma and adenocarcinoma (Fig. 1). In most sarcomatoid mesotheliomas it was difficult to distinguish the cancer tissue from the surrounding stroma (Fig. 1b,e).

The results of the scoring of the hyaluronan stainings are summarized in Fig. 2, Table 1. Hyaluronan staining levels in mesothelioma tumor cells were highly variable (Fig. 2a). Most of the adenocarcinoma cells were negative or contained 6-25% HA positive cells (Fig. 2a). The staining intensity of the cancer cells in most mesotheliomas was weak or moderate, while in the adenocarcinomas it was weak or negative (Fig. 2b). There was statistically significantly less hyaluronan in malignant adenocarcinoma cells than in mesothelioma cells (p=0.001) (Table 1). However most of the stromal cells were hyaluronan positive both in the mesothelioma and in the adenocarcinoma (Fig. 2c) and the staining intensity was weak or moderate (Fig. 2d) with no statistical significance.

Table 1. Comparison of staining analyses of mesotheliomas and adenocarcinomas.

	Epithelial tissue		Stromal tissue	
	positive	negative	positive	negative
HABR				
Mesothelioma	16 (52%)	15 (48%)	22 (88%)	3 (12%)
Adenocarcinoma	2 (8%)	22 (92%) ***	22 (100%)	0 (100%)
CD44				
Mesothelioma	22 (69%)	10 (31%)	16 (48%)	17 (52%)
Adenocarcinoma	10 (42%)	14 (48%)	18 (72%)	7 (28%)
HAS1				
Mesothelioma	10 (30%)	23 (70%)	3 (10%)	28 (90%)
Adenocarcinoma	14 (56%)	11 (44%)	2 (9%)	20 (91%)
HAS2				
Mesothelioma	18 (55%)	15 (45%)	1 (3%)	28 (93%)
Adenocarcinoma	14 (58%)	10 (42%)	3 (13%)	20 (87%)
HAS3				
Mesothelioma	10 (30%)	23 (70%)	2 (7%)	27 (93%)
Adenocarcinoma	11 (46%)	13 (54%)	3 (13%)	20 (87%)

Coverages 3 and 4, and intensities 2 and 3 were classified as positive, and the number of cases for each group were statistically analyzed. Technically failed samples were not included in the analysis. Some samples didn't have any stromal tissue. (***: p<0.001)

Mesotheliomas show tendency for lower CD44 positivity in stromal cells than metastatic adenocarcinomas

Examples of CD44 immunostainings are shown in Fig. 3. Both cancer and stromal cells were typically positively stained for CD44 in epithelioid mesothelioma with moderate intensity (Fig. 3a,d). Most of the sarcomatoid cells were also positive for CD44 (Fig. 3b,e), while the stromal cells showed less intense staining (Fig. 3b). In adenocarcinomas the cancer cells were almost negative for CD44, while the stromal cells showed positive CD44 staining (Fig. 3c,f). In summary, CD44 staining intensity of tumor cells was highest in mesotheliomas, while stromal cells were most intensely stained in adenocarcinomas. The staining was mainly found on the plasma membranes of both epithelial and stromal cells.

The results of the CD44 scoring are presented in Fig. 4, Table 1. The coverage of CD44 staining in most of the cancer cells was 6-50% in epithelioid mesotheliomas (Fig. 4a). Staining pattern for sarcomatoid mesothelioma cells was dualistic. In some lesions most of the cells were stained but in some lesions only 6-25% of the cells were positive for CD44. Most of the adenocarcinoma cells were negative or contained 6-25% CD44 positive cells (Fig. 4a). There were no statistically significant differences between mesothelioma and adenocarcinoma tumor cell CD44 staining intensities (Fig. 4b). Stromal cells in epithelioid mesothelioma lesions were 6-50% positive for CD44 (Fig. 4c). Stromal cells in adenocarcinoma were more frequently positive for CD44 (Fig. 4c), while stromal intensities for CD44 were weak or moderate in mesotheliomas (Fig. 4d). The difference between stromal cells in mesothelioma and in adenocarcinoma CD44 staining was not statistically significant ($p = 0.07$).

Immunostainings of HAS1, HAS2 and HAS3 do not show significant differences between mesothelioma and adenocarcinoma cases

Figure 5 shows examples of HAS1-3 immunostainings. In most of the cases stromal cells were weakly stained or negative for all HAS isoenzymes in both mesotheliomas and adenocarcinomas (Fig. 5a-i). In adenocarcinomas part of the cancer cells were positive for all HAS isoforms (Fig. 5i). Also, a small proportion of cells were positive for HAS1 and HAS2 in epithelioid mesotheliomas (Fig. 5a) and adenocarcinomas (Fig. 5c). In sarcomatoid mesotheliomas the cancer cells were

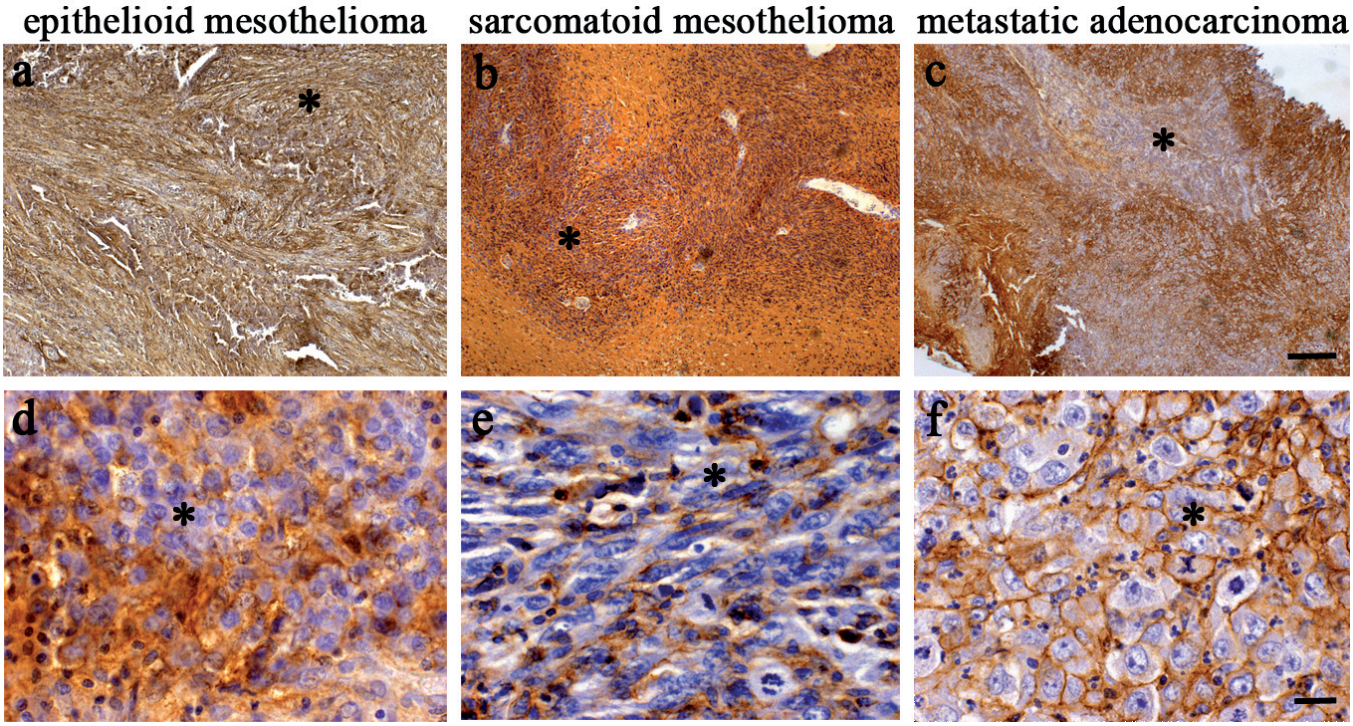

Fig. 1. Hyaluronan staining in epithelioid and sarcomatoid mesotheliomas and metastatic adenocarcinoma. Sections of epithelioid mesothelioma **(a, d),** sarcomatoid mesothelioma **(b, f)** and metastatic adenocarcinoma **(c, f)** were stained with bHABC to detect hyaluronan. Nuclei are stained with haematoxylin (blue) in all of the sections and hyaluronan is shown in brown (DAB). Cancer cells in epithelioid mesothelioma were positive for hyaluronan and stroma was strongly positive **(a, d)**. There was hyaluronan in stroma of sarcomatoid mesothelioma samples and cell surface of cancer cells **(b, e)**. In adenocarcinomas cell surface hyaluronan intensity was typically strong or moderate **(f)** but stromal hyaluronan content was usually high **(c)**. Asterisks indicate the tumor cells. Scale bars: a-c, 200 μm; d-f, 20 μm.

weakly stained for all HAS's (Fig. 5b,e,h), while the overall staining pattern for HAS's was moderate in epithelioid mesotheliomas (Fig. 5a,d,g) and adenocarcinomas (Fig. 5b,e,h). In brief, the staining intensities of HAS isoenzymes were relatively low in all cases and did not show any specific tendency between the two lung cancer types. The staining pattern of all HAS isoforms was typically granular or diffuse, and mostly intracellular with occasional plasma membrane positivity.

The results of HAS scoring are presented in Table 1. There was no statistically significant difference in HAS stainings between adenocarcinoma and mesothelioma samples (Table 1). There was a correlation between HAS2 and HAS3 staining levels in mesothelioma cells, while in stromal cell the levels of HAS1 and HAS2 were correlated. There was no correlation between HASes and CD44 or hyaluronan levels in mesothelioma samples. All three HAS's correlated with each other in adenocarcinoma cells. Levels of HAS3 staining in stromal cells correlated with both HAS1 and HAS2. Stromal CD44 was correlated with adenocarcinoma HAS1 level and slightly with HAS3 (Table 1).

Discussion

Mesothelioma is one of the most aggressive tumors, and its differentiation from less malignant cancer types in lung is highly important for diagnostic purposes. However, the differential diagnosis between malignant mesothelioma and metastatic lung adenocarcinoma is challenging, and often relies on immunohistochemical studies, with no specific individual immunomarkers available. Hyaluronan is one of the potential markers (Azumi et al., 1992; Afify et al., 2005) but differences in methodology have resulted in contradictory results. To refine this process further, we studied 39 epithelioid and sarcomatoid mesotheliomas and 25 adenocarcinoma

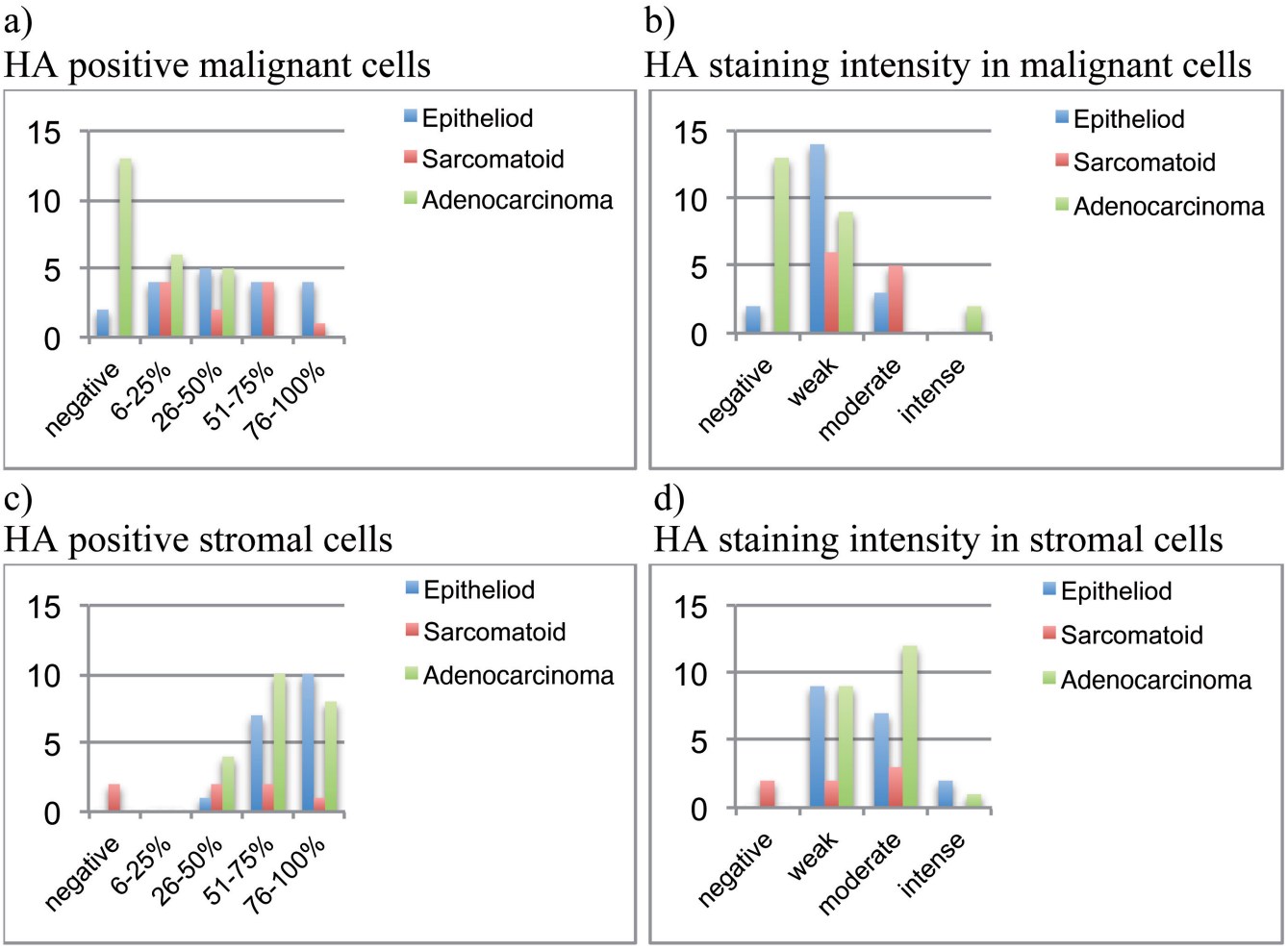

Fig. 2. Comparison of hyaluronan stainings in epithelioid and sarcomatoid mesothelioma, and adenocarcinoma tissue sections. The proportion of tumor cells and stroma positive for hyaluronan in different case groups.

cases by staining the most essential molecules in hyaluronan metabolism, hyaluronan synthesizing enzymes (HAS1-3), CD44 and hyaluronan.

Tumor cell-associated hyaluronan as a potential marker to distinquish between mesotheliomas and adenocarcinomas

Hyaluronan is an important prognostic factor in many epithelial carcinomas (Tammi et al., 2008), accumulating both around tumor cells and in the surrounding stroma. A couple of studies suggest that hyaluronan is a potential diagnostic indicator in mesothelioma, associated with higher tissue content of hyaluronan (Kanomata et al., 2005), cytological staining (Welker et al., 2007), as well as with elevated levels of secreted hyaluronan in pleural effusions or serum of mesothelioma patients (Thylen et al., 1999, 2001). Additionally, tumor associated hyaluronan is suggested to act as a potential tool to differentiate mesotheliomas from adenocarcinomas (Azumi et al., 1992; Afify et al., 2005). The results of this study show that epithelial hyaluronan accumulation is significantly higher in mesotheliomas as compared to metastatic lung adenocarcinomas with mainly negative staining of carcinoma cells. This is in line with previous findings of lung adenocarcinomas with low percentage and low staining intensity of hyaluronan-positive cells (Pirinen et al., 2001). However the results of this work did not show any significant differences in stromal hyaluronan content between adenocarcinoma and mesothelioma. Overall the results of this work show that high hyaluronan content of tumor cells is associated with malignant mesothelioma. This is supported also by finding that hyaluronan increases malignant properties of mesothelioma cells (Li and Heldin 2001). Overall these results support the fact that high tumor content of hyaluronan is associated with poor prognosis (Tammi et al., 2008).

Stromal cell positivity for CD44 is lower in mesotheliomas as compared to adenocarcinomas

CD44 immunostainings have shown decreased CD44 levels in many tumors arising from epidermal keratinocytes, like squamous cell carcinomas and basal cell carcinomas (Karvinen et al., 2003), but increased stromal CD44 acts as a significant prognostic factor in adenocarcinomas like breast cancer (Auvinen et al., 2013). The overexpression of CD44 variant (CD44v6) in rat colon carcinoma cells induces their invasion potential

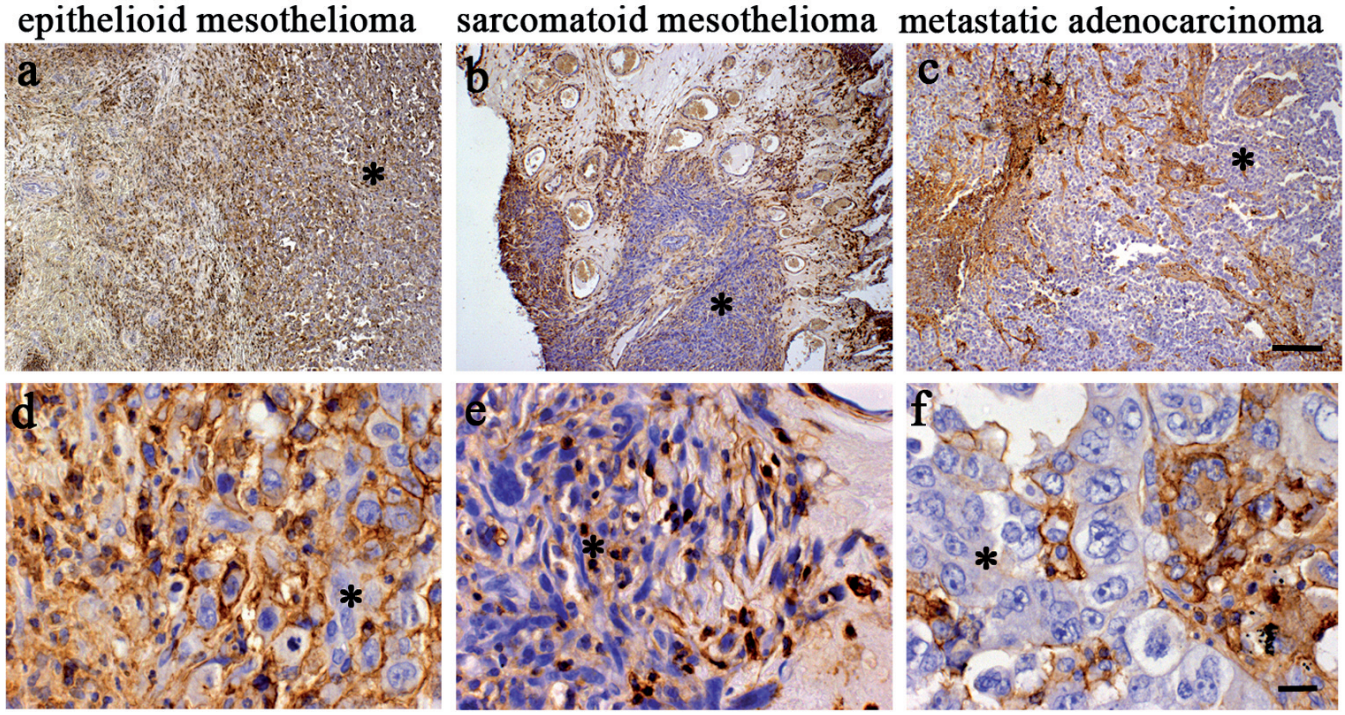

Fig. 3. CD44 immunostaining in mesotheliomas and metastatic adenocarcinomas. Sections of epithelioid mesothelioma **(a, d),** sarcomatoid mesothelioma **(b, e)** and metastatic adenocarcinoma **(c, f)** were stained with Hermes 3 to detect CD44 (brown). Nuclei are stained with haematoxylin (blue). In epithelioid mesotheliomas more than half of the cancer cells were positive for CD44 **(a, d)**. There was less intense staining for CD44 on stromal cells **(a, d).** Sarcomatoid cells were mostly positive for CD44 **(b, e)** and some of the surrounding stromal cells were weakly positive **(b, e)**. Cancer cells in adenocarcinoma were negative for CD44 **(c, f)** and most of stromal cells showed moderate CD44 expression **(c, f)**. Asterisks indicate the tumor cells. Scale bars: a-c, 200 μm; d-f, 20 μm.

(Gunthert et al., 1991), which may be due to modification of interactions and signaling between growth factor receptors and cell cytoskeleton. CD44 interaction with hyaluronan is an important regulator of mesothelioma cell migration and proliferation (Hanagiri et al., 2012), and CD44 has been suggested to act as a potential marker to distinquish mesothelioma from adenocarcinoma (Attanoos et al., 1997). Additionally, the capability of CD44 to bind matrix metalloproteinases on the cancer cell surface increases the invasive potential of cancer cells (Ponta et al., 2003). Thus the role of CD44 is conflicting, because its function in hyaluronan binding and signaling most probably enhances cancer cell survival (Heldin et al., 2008), but it may also mediate hyaluronan endocytosis (Tammi et al., 1998), which may instead lead to decreased hyaluronan content around tumor cells. Our results show that there is a tendency for lower immunostaining of hyaluronan receptor CD44 in stromal cells of mesothelioma as compared to adenocarcinomas. Interestingly, the lower CD44 positivity of stromal cells in mesotheliomas in comparison to adenocarcinomas may be related to the higher hyaluronan levels in mesotheliomas.

Hyaluronan synthases

Hyaluronan synthases play a crucial role in accumulation of hyaluronan in breast cancer and increased hyaluronan synthases correlate with poor prognosis (Auvinen et al., 2013, 2014). However, also posttranslational factors regulating activity of hyaluronan synthases, like availability of sugar precursors (Jokela et al., 2011; Tammi et al., 2011; Rilla et al., 2013) as well as expression and activity of hyaluronan degrading enzymes, hyaluronidases (Nykopp et al., 2009, 2010) have an impact on the accumulation of hyaluronan in tissues. HAS staining intensity is typically increased in epithelial cancers as compared to normal epithelia with low levels of HAS staining (Nykopp et al., 2009, 2010). However, both normal

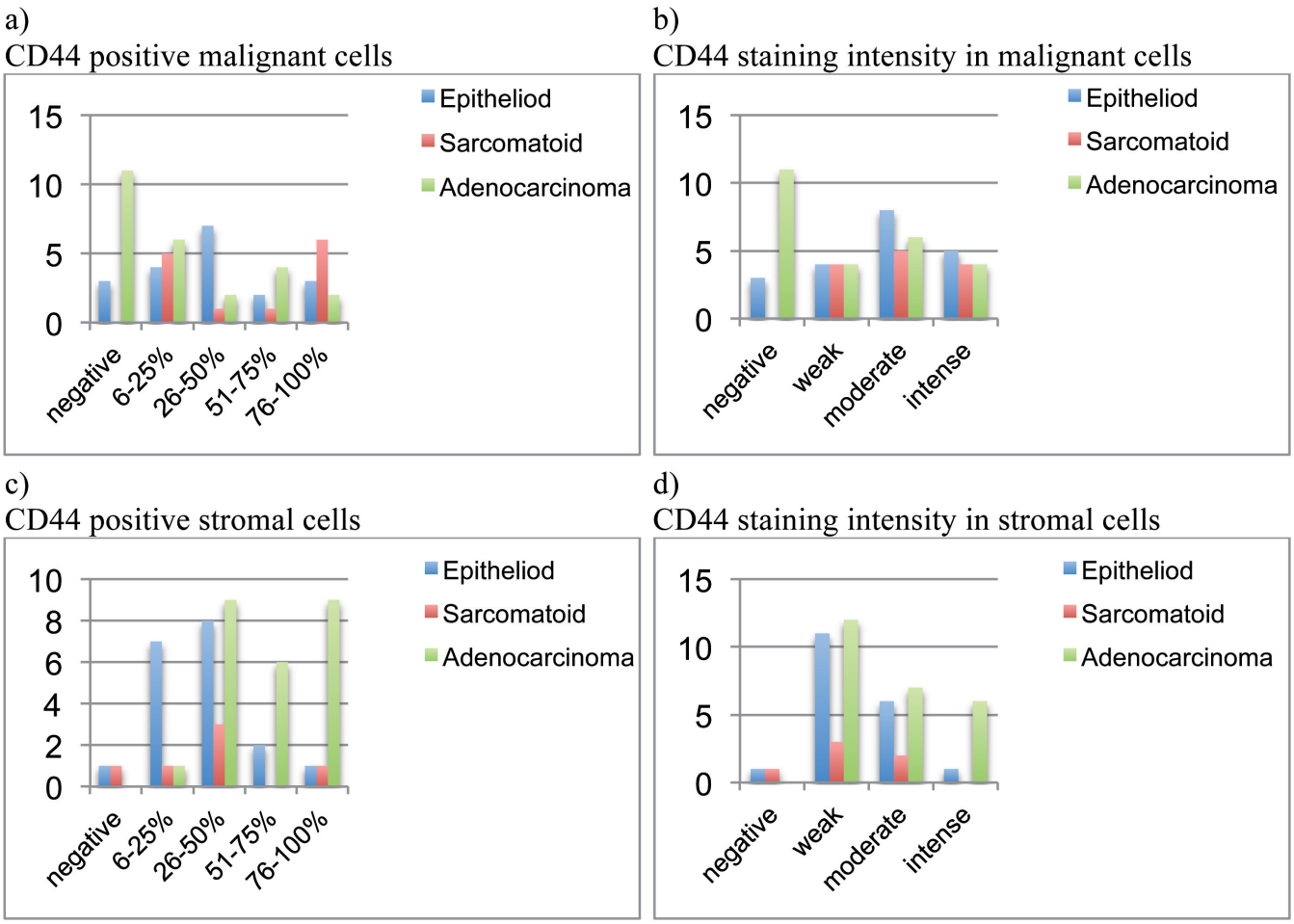

Fig. 4. CD44 expression in epithelioid and sarcomatoid mesothelioma, and adenocarcinoma. The proportion of tumor and stromal cells positive for CD44 in different case groups.

mesothelial cells (Liu et al., 2004) and malignant mesothelioma cells (Liu et al., 2004) are known to secrete high levels of hyaluronan and express all HAS isoforms. Furthermore, overexpression of all HAS isoforms has been reported in mesothelioma sections (Kanomata et al., 2005). It has been suggested that growth factors released by mesothelioma cells can stimulate surrounding normal mesothelial cells and fibroblasts to secrete more hyaluronan (Asplund et al., 1993; Asplund and Heldin 1994). This kind of regulation could be due to either pre-or post-translational activation of hyaluronan synthases. These complex regulatory steps complicate the predicting of the actual activity levels of existing hyaluronan synthases when visualized by immunostainings. The results of this study showed no statistically significant differences in HAS stainings in mesotheliomas as compared to adenocarcinomas, but as discussed above, we cannot exclude the putative post-transcriptional regulation of HAS activity.

Final conclusions

The main finding of this work is that mesotheliomas show significantly higher hyaluronan positivity around tumor cells and a tendency for lower stromal cell positivity for CD44 than metastatic adenocarcinomas. The results of this study support the previous findings of hyaluronan and CD44 as potential markers to differentiate adenocarcinomas from mesotheliomas. However, these markers alone are not specific enough to be used as diagnostic tools, but they could act as markers together with other specific markers. In any case, the results of this work provide one step towards better understanding of hyaluronan metabolism in lung

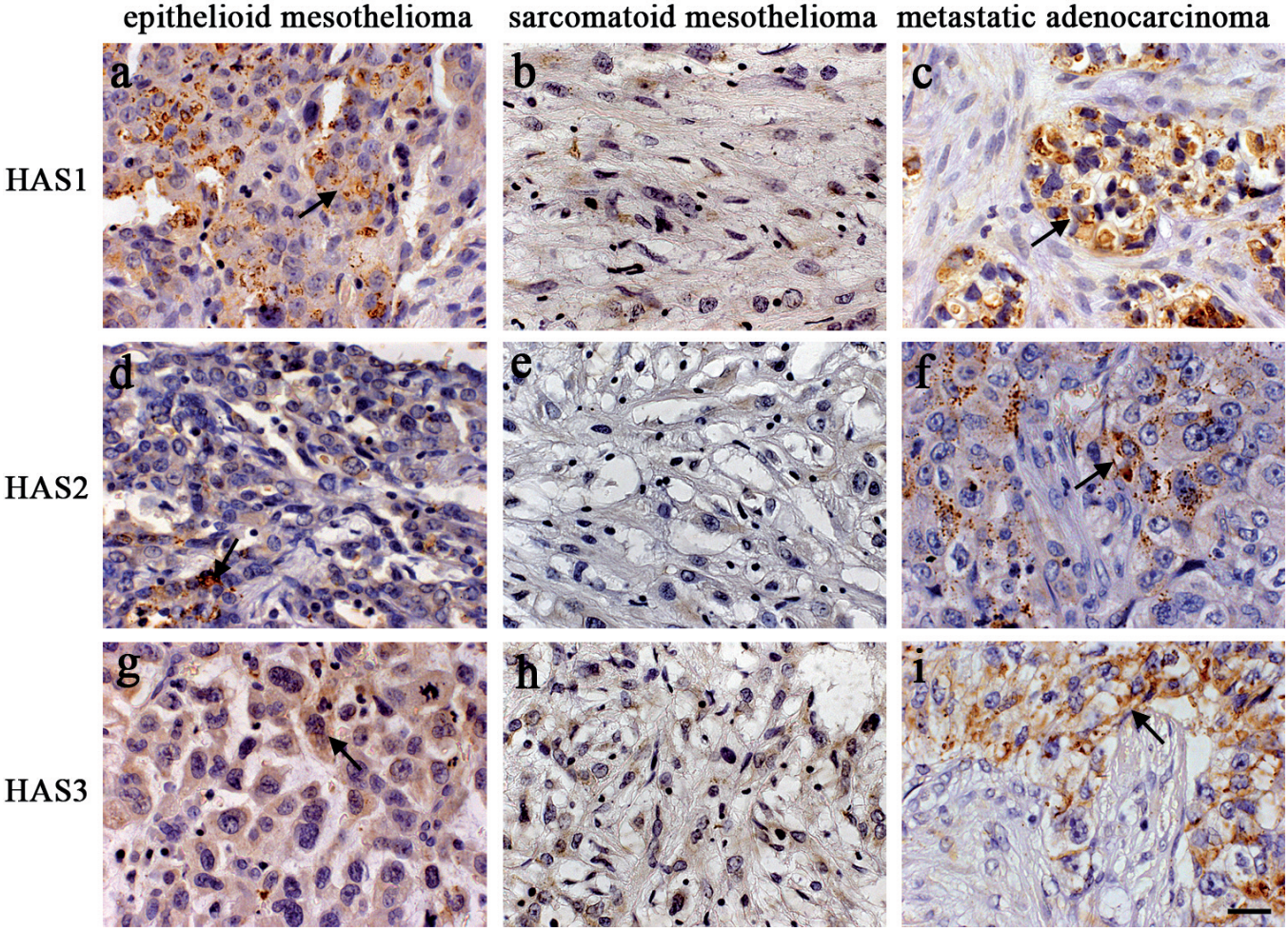

Fig. 5. Hyaluronan synthase immunostaining in mesotheliomas and metastatic adenocarcinomas. Sections of epithelioid mesothelioma **(a, d, g)**, sarcomatoid mesothelioma **(b, e, h)** and metastatic adenocarcinoma **(c, f, i)** were stained with HAS1 **(a, b, c),** HAS2 **(d, e, f)** and HAS3 **(g, h, i)**. All HAS's have low staining intensity and percentage in epithelioid mesotheliomas and adenocarcinomas. There were some epithelial cells with moderate staining intensity for HAS1 **(c)** and for HAS3 **(i)** in adenocarcinomas. Sarcomatoid mesotheliomas were mostly negative for HAS's. Stromal cells were negative for HAS's in all sample groups. Arrows point HAS-positive tumor cells in all panels. Scale bar: 20 μm.

cancers.

Acknowledgements. Special thanks are due to Eija Rahunen and Kari Kotikumpu for expert technical assistance. Authors would like to thank prof. Raija Tammi for valuable comments on the manuscript. This work was supported by the Academy of Finland, Sigrid Juselius Foundation, Paavo Koistinen foundation, North-Savo Cancer Fund and the Spearhead Funds from the University of Eastern Finland (Cancer Center of Eastern Finland).

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Accepted February 25, 2016