

Bone morphogenetic protein 4 expression in multiple normal and tumor tissues reveals its importance beyond development

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Bone morphogenetic proteins (BMPs) are extracellular signaling molecules that belong to the transforming growth factor β (TGF β) superfamily and are known to regulate cell proliferation, differentiation and motility, especially during development. BMP4 has an indispensable role in vertebrate development while limited information on BMP4 expression and function exists in adult tissues. Nevertheless, its contribution to cancer development and progression has gained increasing interest in recent years. Functional studies, especially in breast cancer, have implicated BMP4 both in inhibition of cell proliferation and in promotion of cell migration and invasion. To gain an insight into the function of BMP4 in normal and cancer tissues, BMP4 protein expression levels were analyzed by immunohistochemistry in 34 different normal organs/tissues, 34 different tumor types and finally in 486 breast cancer samples where possible associations between BMP4 and clinicopathological parameters were statistically evaluated. In over 20% of normal and malignant tissues, BMP4 was expressed at high level. Strong expression was observed particularly in some normal epithelial cells, such as bladder and stomach, and in squamous cell carcinomas. In breast cancer, strong BMP4 expression was detected in 25% of patients, and was associated with low proliferation index and increased frequency of tumor recurrence. Taken together, BMP4 is expressed in a subset of normal adult tissues and is likely to contribute to tissue homeostasis. However, in tumors, BMP4 expression levels vary considerably, implying diverse roles in different tumor types. This role is biphasic in breast cancer as BMP4 expression is linked to reduced proliferation and increased recurrence, thus corroborating our previous *in-vitro* functional data.

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Bone morphogenetic protein 4 (BMP4) is an extracellular signaling molecule that belongs to the family of bone morphogenetic proteins (BMPs) comprising the largest subfamily of the transforming growth factor β (TGF β) superfamily.¹ BMPs were originally named according to their bone inducing function, but they are also significant regulators of development.^{2,3} BMP4 is crucial for normal

development as the homozygous deletion of *Bmp4* in mice is embryonic lethal.⁴ The mouse embryos die between 6.5 and 9.5 days postcoitum and suffer from defects in gastrulation and mesoderm formation.^{3–5} *Bmp4* expression is also detected later during organogenesis of the kidney, tooth, lung and gut as well as during skeletogenesis, limb patterning and mammary gland development.^{3,6,7}

On a cellular level, BMPs are able to modulate cell proliferation, differentiation, survival, migration and even cell fate.³ This enormous pleiotropy stems from the intensive regulation of this signaling pathway.^{8,9} BMPs are synthesized as large precursor monomer proteins that are subsequently cleaved, dimerized and secreted as mature cytokine dimers

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outside the cell, or they may alternatively be stored in vesicles inside the cell.¹⁰ Mature BMP dimers bind to their specific transmembrane receptors to activate intracellular SMAD proteins that regulate target gene transcription.⁸ The BMP-SMAD pathway is also able to interconnect with other signaling pathways, and BMPs are known to activate the MAP kinase pathways independent of the SMAD proteins.^{9,10}

The embryonic lethality caused by the loss of *Bmp4* has impeded studies of BMP4 function in adult mammals. There have only been a few studies on the function of BMP4 in adult tissues and disease states other than cancer. BMP4 is involved in skeletal repair and the bone regeneration processes.¹¹ In normal ovaries, BMP4 is expressed in a cell type-specific manner and is involved in dynamic changes during the ovarian cycle.¹² *Bmp4* is expressed in the endocrine pancreas regulating insulin metabolism, and in the spleen during stress-induced erythropoiesis.^{13,14} Furthermore, BMP4 has been implicated in the idiopathic pulmonary fibrosis of the lung.¹⁵ Increased BMP4 expression has been observed in esophagitis and Barrett's esophagus where BMP4 induces the metaplastic transformation of squamous epithelial cells to columnar cells.¹⁶ These examples indicate that BMP4 clearly has a specific role in various processes in adult tissues and organs although comprehensive data on this issue are currently lacking.

BMP4 has been studied in several cancer types that originate from various tissues.¹⁷ In these studies, BMP4 has been functionally linked to the reduced growth of prostate, breast and pancreatic cancer cells, the increased migration and invasion of breast, colorectal, ovarian, melanoma and pancreatic cancer cells, the induction of the epithelial-mesenchymal transition in ovarian and pancreatic cancer cells and to lung cancer cell senescence.^{18–25} Interestingly, *BMP4* has also recently been identified as a new susceptibility locus for colorectal cancer.²⁶ *BMP4* expression has been detected in various cancer cell lines and tumor cells, but often, the number of samples included in these studies has been low. BMP4 protein expression has also been detected in tumor samples from colorectal, gastric, hepatocellular, ovarian and renal carcinomas as well as in squamous cell carcinomas of the head and neck and melanomas.^{19,20,27–31} Yet, only a handful of studies have explored BMP4 expression within a particular tumor type. For example, in breast cancer, the information is truly limited.³² We have previously shown that *BMP4* is strongly expressed in breast cancer cell lines and primary breast tumors.^{23,33} Furthermore, we proposed a bidirectional function for BMP4 as *in vitro* it reduced cancer cell proliferation and yet was able to simultaneously increase the migration and invasion of breast cancer cells.²³ Currently, no studies exploring BMP4 protein expression in breast tumors have been conducted.

In this work, we first systematically evaluated BMP4 protein expression in multiple normal and malignant tissues throughout the human body. Second, we studied BMP4 expression and its possible clinical significance in a large set of tumor tissues, including both ductal and lobular carcinomas, from breast cancer patients with extensive clinical data. BMP4 protein expression was visualized by immunohistochemistry in 34 different normal tissues, 34 different tumor types and 486 breast tumor samples.

Materials and methods

Tissue Microarrays

Three different tissue microarrays were used in this study. The normal human tissue array (MNO961) and the multiple organ tumor tissue array (MC2082) were obtained from US Biomax, Inc. (Rockville, MD). The tissue microarrays contained formalin-fixed paraffin-embedded tissue samples with a core diameter of 1–1.5 mm. The normal tissue array contained 96 tissue specimens from 34 different types of organs or anatomical sites either 1 sample (bone marrow and parathyroid), 2 samples (eye, pituitary gland, skin, spinal cord and spleen), 3 samples (adrenal gland, bladder, breast, cerebellum, cerebral cortex, colon, esophagus, fallopian tube, heart, liver, lung, ovary, pancreas, placenta, prostate, rectum, small intestine, stomach, striated muscle, testis, thymus, thyroid, tonsil, ureter, uterus cervix and uterus endometrium) or 6 samples (kidney) of each tissue type.

The tumor tissue array contained 208 tumor samples from 34 tumor types (including subtypes) and 20 different organs or anatomical sites. Tumors samples included eight cases each of adenocarcinoma of the stomach, colon, pancreas, endometrium and prostate, eight hepatocellular carcinomas, eight lymphomas of the spleen, eight seminomas, eight transitional cell carcinomas of the bladder, four esophagus adenocarcinomas and squamous cell carcinomas, four each of lung squamous cell carcinomas, adenocarcinomas, large cell and small cell carcinomas, four astrocytomas, four glioblastomas, four thyroid papillary and follicular carcinomas, four Hodgkin and non-Hodgkin lymphomas, eight ductal and lobular breast carcinomas, eight ovarian adenocarcinomas, four ovarian germ cell and stromal tumors, eight kidney clear cell and granular cell carcinomas, eight squamous cell carcinomas of the uterine cervix, eight squamous cell carcinomas of the head and neck, eight squamous cell carcinomas of the skin, four fibrosarcomas of the skin, four liposarcomas and eight malignant melanomas.

The breast cancer tissue microarray contained 243 invasive ductal carcinomas and 243 invasive lobular carcinomas that are part of a previously published

larger study.^{33,34} Each tumor sample was represented by a single core biopsy with a diameter of 1 mm on the tissue microarray. The patient records contained information on clinicopathological parameters, primary treatments, recurrences and survival. The sites and times of recurrences as well as the patient survival information were updated on 30 October 2009. The mean age of the patients at the time of operation was 61 years (range 31–93). From the 486 patients, 83% were ER positive and 66% were PR positive (ER/PR status was missing in 2.5% of the cases). The grade distribution included 40% grade 1, 46% grade 2 and 14% grade 3 with 0.2% of the cases not having available data. The pathological tumor stage was pT1 in 61%, pT2 in 30% and pT3-4 in 9% of the patients with data missing in 0.4% of the cases. The pathological lymph-node stage was negative (pN0) in 56% of the patients and positive (pN1-2) in 38% of the patients with no available information for 6% of the cases. The pathological metastasis stage was positive (pM1) for 3%, negative for 96% and missing for 1% of the patients. Altogether, 12% of the patients were ERBB2 positive (data missing in 5% of the cases). The DNA content was diploid for 51% of the patients and either aneuploid or multiploid for 33% of the patients with 16% of the cases not having available data. The proliferation index information was obtained from patient records and had been determined either by flow cytometry or by Ki67 staining depending on the year of diagnosis. For flow-cytometric DNA content analysis, an S-phase fraction of <7% was considered as low, 7–12% as intermediate and >12% as high proliferation rate. For Ki67 staining, <10% positive cells was considered as low, 10–15% as moderate and >15% as high proliferation. Altogether, the proliferation rate was low in 34%, moderate in 23% and high in 19% of the patients with no information available for 24% of the cases. All except one of the patients had their primary tumors surgically removed and the majority of them with axillary clearance. Post-operative radiotherapy was given for 28% of the patients, 7% received adjuvant systemic treatment (endocrine therapy and/or chemotherapy), 37% of the patients were treated with both radiotherapy and systemic treatment, 28% received neither radiotherapy nor systemic treatment and in eight cases no treatment information was available. During the follow-up period, a local cancer recurrence was detected in 11% of the patients and a distant metastasis was found in 37% of the patients. The distribution of the metastasis sites is presented in Supplementary Table 1. The maximum follow-up period was 19.8 years, which contained information on the site and time of tumor recurrence and overall survival time. The use of the tumor samples and patient records in this study was approved by the Ethics Committee of Pirkanmaa Hospital District, the Ministry of Social Affairs and Health, and the National Authority for Medicolegal Affairs.

BMP4 Immunohistochemistry

BMP4 protein expression in tissue samples was studied with immunohistochemistry. Immunostaining was performed using an automated Ventana Benchmark staining system according to manufacturer's instructions (Ventana Medical Systems, Tuscon, AZ). The tissue microarray slides were first deparaffinized and rehydrated, followed by antigen retrieval (heat treatment in 10 mM Tris-HCl, 1 mM EDTA). BMP4 protein was detected with a mouse anti-BMP4 monoclonal antibody (clone 3H2, 1:10 dilution; Chemicon Millipore, Temecula, CA) and an ultraView Universal DAB Detection Kit (Ventana Medical Systems). The slides were counterstained with hematoxylin-eosin. A control with no BMP4 antibody showed no staining. To further verify the specificity of the BMP4 antibody, immunocytochemical staining of breast cancer cell lines with high (HCC38) and low/no (HCC1954) BMP4 mRNA expression²³ was performed and revealed strong and no expression, respectively.

The entire tissue core for each sample was evaluated by pathologists, and the BMP4 staining was classified as negative, weak diffuse, moderate diffuse or strong granular staining. For normal tissues, each sample was classified based on the staining pattern observed in the majority (at least 75%) of the cells except that samples with at least 10% of the cell showing granular staining were classified as strong granular. Data from different tissue compartments, eg, epithelium and stroma, were recorded separately when applicable. For cancer samples, the same scoring criteria were applied but only tumor cells were evaluated. The possible presence of leukocytes with high BMP expression was recorded separately and did not influence the scoring of the corresponding normal tissue or tumor samples.

Statistical Analysis

The significance of BMP4 expression in primary breast tumors was evaluated using statistical analyses (SPSS program, version 11.01; SPSS Inc., Chicago, IL). The associations of BMP4 expression with standard clinicopathological parameters and tumor recurrences were analyzed using Fisher's exact test. The Kaplan-Meier method and the log-rank test were applied to estimate the differences between the BMP4 expression groups in the rate of first local and distant recurrences as well as the overall survival time, breast cancer-specific survival time and disease-free time.

Results

The Classification of BMP4 Expression in Tissue Samples

BMP4 protein expression was determined in formalin-fixed paraffin-embedded normal and tumor tissue

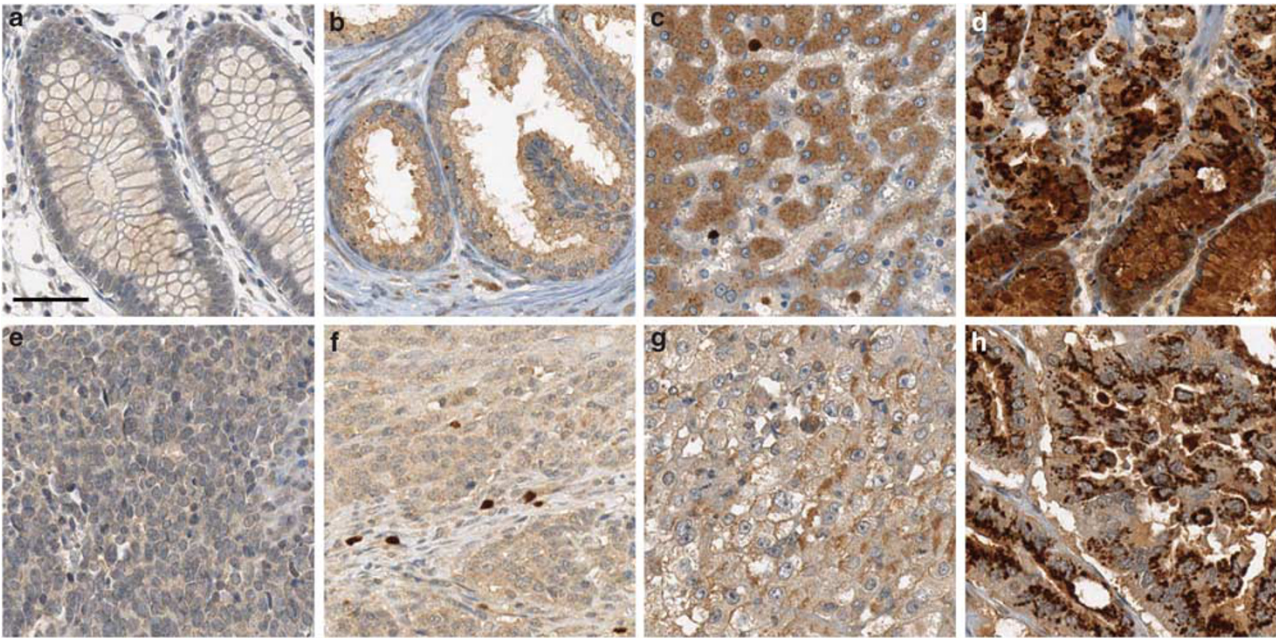


Figure 1 BMP4 expression in (a–d) normal tissue samples and (e–h) tumor samples. Representative examples of negative BMP4 expression in (a) normal colon and (e) small cell carcinoma of the lung, low diffuse staining of BMP4 in (b) normal prostate and (f) stomach adenocarcinoma, moderate diffuse staining in (c) normal liver and (g) endometrioid adenocarcinoma of the uterus, and strong granular staining in (d) normal stomach and (h) lung adenocarcinoma are shown. Liver tissue macrophages (Kupffer cells) are strongly stained in the liver, and leukocytes are strongly stained in the tumor sample of the stomach. The scale bar in (a) indicates 50 μm and is the same for all images.

samples by immunohistochemistry. BMP4 expression was classified into four different staining categories: negative, weak diffuse, moderate diffuse and strong granular staining (Figure 1). In the diffuse staining category, BMP4 expression was detected evenly throughout the cytoplasm, whereas in the strong granular staining category, distinct granules were evident in the cytoplasm possibly suggesting a secretory expression pattern. Thus, the BMP4 staining could also be categorized into non-granular and granular patterns.

BMP4 Expression in Normal Tissues

BMP4 protein expression was first explored in the normal tissues. Altogether, of the 34 tissues or organ sites evaluated, 9 (26%) were considered as negative for BMP4 expression and 5 (15%) expressed BMP4 diffusely at a low level (Table 1; Figure 1a and b). Moderate diffuse staining was detected in two organs, the liver and parathyroid gland (Figure 1c).

Strong granular BMP4 expression was detected in 8 out of the 34 tissues (24%) (Table 1; Figure 1d). In seven organs, the granular expression was detected particularly in the epithelial cells of the tissue. BMP4-positive granules were identified in the transitional epithelium of the bladder and ureter. Likewise, the stratified squamous epithelium was positive for BMP4 granules in the oral cavity and ectocervix. In the alimentary canal, the stratified squamous epithelium of the esophagus also strongly expressed BMP4. In the stomach, especially strong

staining was detected in the epithelial cells of the surface mucosa and the chief cells of the base segment of the gastric glands, whereas the parietal cells clearly showed weaker staining. BMP4-positive granules were also observed in the lung cells lining the alveoli. In non-epithelial tissues, granular BMP4 expression was identified in the red pulp area of the spleen.

Altogether, 15% (5/34) of the tissues could not be clearly classified into the four main staining categories (Table 1). A more specific staining pattern for BMP4, denoted as punctuated staining, was detected in the skeletal and cardiac muscles. In the cerebellum, cerebral cortex and spinal cord, BMP4 expression was detected only in cells that most likely represent the microglia of the central nervous system.

Five tissues and/or organ sites (15%) showed variable staining between the samples. For example, granular BMP4 staining was identified in some, but not all samples, from tissues of the fallopian tube, kidney, endometrium and skin. In the kidney, strong BMP4 expression was detected in the collecting ducts, diffuse staining was detected in the proximal tubules and negative staining was observed in the glomeruli. Additionally, in the skin, granular BMP4 staining was observed particularly in the hair follicles.

Interestingly, throughout the tissue samples, strong BMP4 expression was observed in leukocytes (such as neutrophilic granulocytes in the placenta) and often in the tissue-specific macrophages (eg, the Kupffer cells of the liver) (Figure 1c). Overall, 21 out of the 34 tissue sites had positively stained

Table 1 BMP4 expression in 34 organs or tissues

Organ/tissue	BMP4 staining				Special pattern	Pos LC ^a
	Neg	Low	Moderate	Granular		
Adrenal gland	×					×
Bone marrow ^b	×					
GI-Small intestine	×					×
GI-Colon	×					×
GI-Rectum	×					×
Ovary	×					
Pancreas	×					×
Thymus	×					×
Thyroid	×					×
Eye ^c	×					
Breast	×					
Placenta	×					×
Prostate	×					
Testis	×					
Liver		×				×
Parathyroid ^b		×				×
Bladder			×			×
GI-Esophagus			×			×
GI-Stomach			×			
Lung			×			×
Spleen ^c			×			×
Tonsil			×			
Ureter			×			
Uterus-cervix			×			×
Heart ^d					×	
Striated muscle ^d					×	
Cerebellum					×	×
Cerebral cortex					×	×
Spinal cord ^c					×	×
Pituitary gland ^c	×	×				
Uterus-endometrium	×		×			
Fallopian tube	×	×		×		×
Kidney ^e	×	×		×		×
Skin ^c	×	×		×		×

BMP4 staining was classified as negative, low diffuse, moderate diffuse or strong granular. In the special pattern group, BMP4 staining was not classified into any of the four main groups.

^aBMP4 expression in leukocytes.

^bNumber of samples, $n = 1$.

^cNumber of samples, $n = 2$.

^dPunctuated staining pattern.

^eNumber of samples, $n = 6$.

leukocytes (Table 1). Yet, due to the intensive BMP4 staining, the exact identification of the leukocyte type was not possible. Interestingly, the lymphatic cell population in the tonsils was negative for BMP4 expression.

BMP4 Expression in Tumor Tissues

Next, we examined the BMP4 expression in the multiple organ tumor tissue microarray. Half of the tumor samples were either BMP4 negative (14%) or

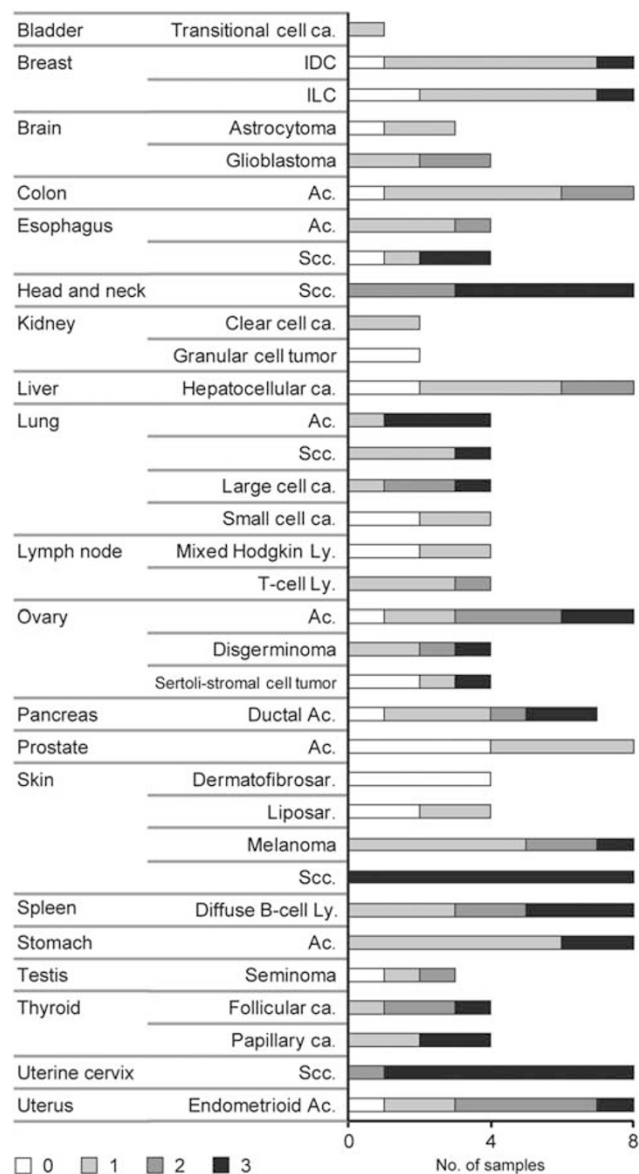


Figure 2 BMP4 expression in multiple tumor tissue arrays. Numerals indicate the strength of BMP4 expression (0 = negative, 1 = low, 2 = moderate and 3 = strong granular staining). ca., carcinoma; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; Ac., adenocarcinoma; Scc., squamous cell carcinoma; Ly., lymphoma; Sar., sarcoma.

expressed BMP4 at low level (37%) (Figures 1e, f and 2). Moderate diffuse staining was detected in 14% of the samples and 22% had a strong granular BMP4 expression pattern (Figures 1g, h and 2). In 13% of the samples, the BMP4 status could not be determined due to missing or unrepresentative samples.

The most notable common feature among the samples was the strong granular BMP4 expression in tumors arising from squamous cell epithelia particularly in the squamous cell carcinomas of the head and neck, skin and uterine cervix and to a lesser degree in the esophagus and lung (Figure 2).

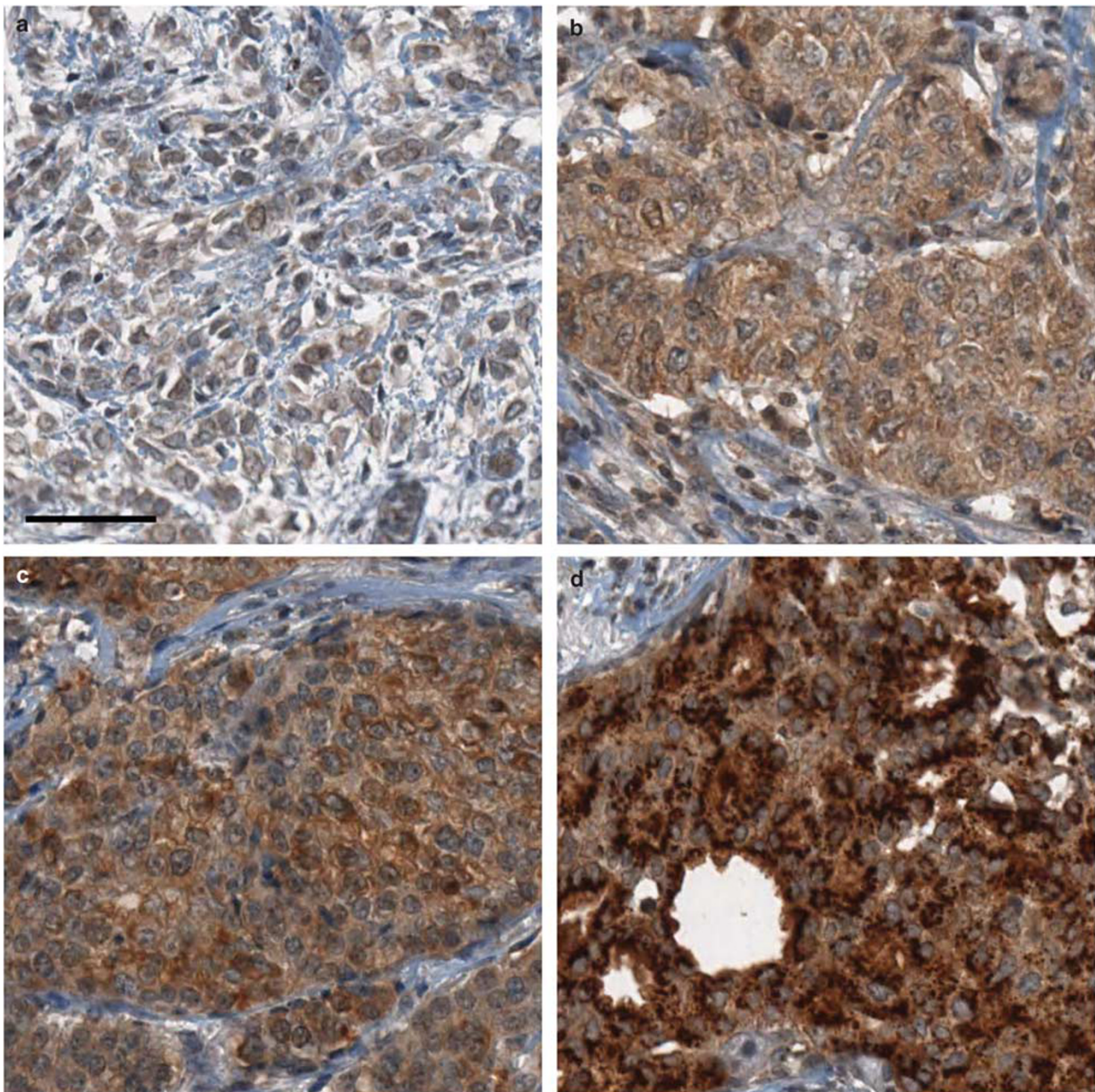


Figure 3 BMP4 expression in a breast cancer tissue microarray. Representative examples are shown for (a) negative, (b) weak diffuse, (c) moderate diffuse and (d) strong granular staining of BMP4. The scale bar in (a) indicates 50 μm and is the same for all images.

In general, roughly half of the tumor types examined had BMP4 expression pattern that ranged from a negative/low to a strong level (Figure 2). In these tumor types, strong granular BMP4 expression was detected in less than half of the samples within a given cancer type (eg, ovary, pancreas and uterus). In approximately one-third of the tumor types, no BMP4 granular staining was observed (eg, brain, colon and prostate) (Figure 2).

Nine organs were represented by more than one histological tumor type in our survey. Specific differences in BMP4 expression within tumor subtypes were detected in the lung and skin. In

the lung, 42% (5/12) of the non-small cell lung carcinoma samples (adenocarcinoma, squamous cell carcinoma and large cell carcinoma) had strong BMP4 granular expression, and none were negative. Contrarily, the small cell carcinoma samples were either BMP4 negative or displayed a low expression level (Figure 1e and h). Similarly, in the skin, the expression pattern also varied between tumor types as dermatofibrosarcomas and liposarcomas expressed BMP4 at lower level compared with the melanoma and squamous cell carcinoma samples (Figure 2). In the esophagus, granular expression was not detected in adenocarcinomas but only in

squamous cell carcinomas. In contrast, no drastic differences were observed between tumor subtypes of the breast, ovary or thyroid. In these tumors, BMP4 was expressed in the majority of samples, and the expression patterns, ranging from weak to granular, were represented almost equally. Finally, mainly negative or weak BMP4 expression was detected in astrocytomas, glioblastomas and lymphomas originating from the lymph nodes.

The remaining tumors contained only one histological subtype on the tissue microarray. Nearly, all samples expressed BMP4 in four tumor types (ie, pancreas, spleen, stomach and uterus) even though strong granular BMP4 staining was observed in less than half of the samples. In three cancers, colon, liver and prostate, BMP4 expression was generally weak, and granular staining was not observed. The number of samples analyzed was very low for bladder, testicular and the two types of renal cancer.

Due to the relatively low number of samples for each tumor type, no conclusions were drawn between the expression of BMP4 and the available tumor characteristics. However, the observation that BMP4 expression in astrocytomas was lower than in glioblastomas was noteworthy as glioblastomas are considered to be a more severe form of brain tumor. Among the stromal cells, leukocytes in the tumor tissue were strongly positive for BMP4 similar to the normal tissue samples. Of note, there was no association between the presence of positively stained leukocytes and high BMP4 expression in the tumor tissue.

BMP4 Expression in Breast Cancer

Finally, a breast cancer tissue microarray that included 486 patient samples was utilized to study BMP4 expression. BMP4 expression was determined in 66% (314/486) of the tumors, of which 171 were invasive ductal carcinomas and 143 were invasive lobular carcinomas. The rest of the samples were either missing or unrepresentative. BMP4 expression was found to be negative or weak in 197 (63%), moderately diffuse in 44 (14%) or strongly granular in 73 (23%) of the tumor samples (Figure 3). To study the association of BMP4 expression with standard clinicopathological parameters, the samples were divided into two groups: non-granular BMP4 expression ($n=241$) and granular BMP4 expression ($n=73$). Strong granular BMP4 staining was detected more often in the lobular carcinoma group compared with the ductal carcinoma group (64 vs 36%, $P<0.001$) (Table 2). Patients with tumors containing BMP4 granular staining appeared to have more slowly proliferating cancers as a higher fraction of these tumors had low proliferation indexes compared with the non-granular tumors (52 vs 36%, $P=0.019$) (Table 2). No further statistically significant associations were

Table 2 BMP4 expression and the standard clinicopathological parameters of 314 breast tumor samples.

Variable	Non-granular ($n=241$)		Granular ($n=73$)		P-value
	n	%	n	%	
<i>Tumor type</i>					
ILC	96	40	47	64	<0.001
IDC	145	60	26	36	
<i>pT stage</i>					
pT1	146	61	48	66	0.728
pT2	73	30	20	27	
pT3–pT4	21	9	5	7	
NA	1				
<i>pN stage</i>					
pN0	132	57	42	65	0.578
pN1	89	39	21	32	
pN2	9	4	2	3	
NA	11		8		
<i>pM stage</i>					
pM0	227	95	71	97	0.740
pM1	11	5	2	3	
NA	3				
<i>ER^a</i>					
Positive	199	85	60	82	0.612
Negative	36	15	13	18	
NA	6				
<i>PR^a</i>					
Positive	163	69	48	66	0.562
Negative	72	31	25	34	
NA	6				
<i>Proliferation^b</i>					
Low	67	36	27	52	0.019
Moderate	65	35	8	15	
High	53	29	17	33	
NA	56		21		
<i>ERBB2^a</i>					
Positive	30	13	5	7	0.185
Negative	202	87	65	93	
NA	9		3		
<i>Histological/nuclear grade</i>					
I	81	33	27	38	0.322
II	115	48	37	51	
III	45	19	8	11	
NA			1		
<i>DNA</i>					
Diploid	109	52	35	61	0.227
Aneu/Multiploid	99	48	22	39	
NA	33		16		

NA, not available.

^aER, PR and ERBB2 statuses were determined by immunohistochemistry.

^bProliferation activity was determined by flow cytometry (S-phase fraction <7% low, 7–12% moderate and >12% high), or Ki67 staining (Ki67 <10% low, 10–15% moderate and >15% high).

P-values <0.05 are shown in bold.

found between BMP4 expression and other standard clinicopathological parameters (eg, ER/PR status, grade, TNM stage, DNA content or ERBB2 status).

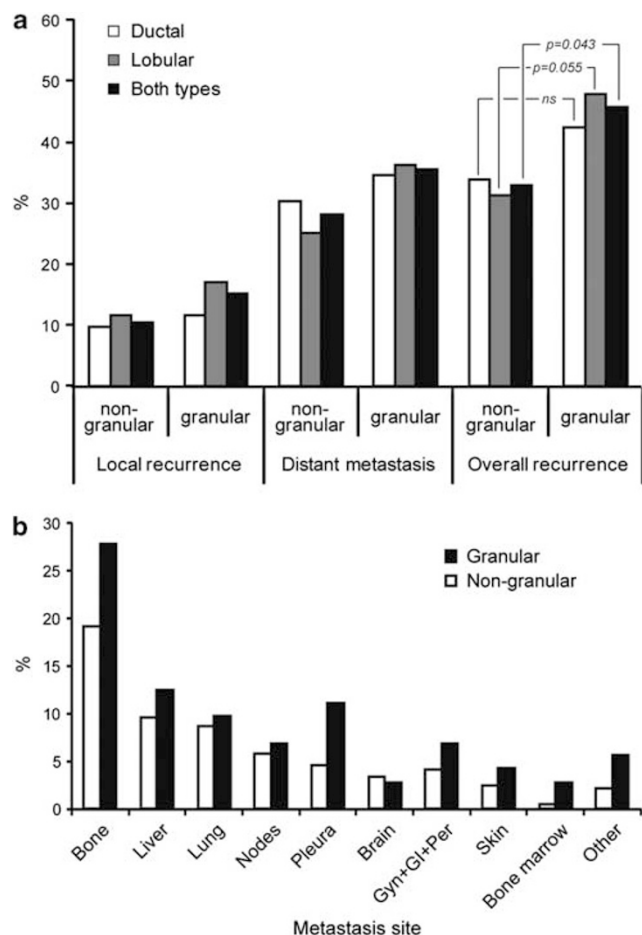


Figure 4 BMP4 expression correlates with breast tumor recurrence. (a) The percentages of patients that suffered from local, distant and overall recurrences are shown. A P -value of <0.05 was considered to be statistically significant (NS, not significant). (b) The percentages of patients with BMP4 granular or non-granular primary tumors that had distant metastases at a particular organ site are shown.

Next, the association between BMP4 expression and tumor recurrence was evaluated. Patients with tumors containing strong granular BMP4 staining suffered from overall tumor recurrence, including both local recurrences and distant metastases, more often than patients with tumors displaying non-granular BMP4 staining (49 vs 33%, $P=0.043$) (Figure 4a). This association was subsequently explored in each tumor type separately. Among lobular carcinomas, the tumor recurrence was even more prominent within patients with granular tumors (48 vs 31%, $P=0.056$) (Figure 4a). Yet, due to the low total number of patients with tumor recurrence, this difference was not statistically significant (Figure 4a). We then explored whether the observed increase in recurrence from tumors with BMP4 granular staining was reflected in particular sites of metastasis (eg, bone, liver, lung, nodes, pleura, brain, gynecological organs, GI tract, peritoneum, skin and bone marrow). In general, patients with tumors displaying BMP4 granular

staining more often showed metastasis at all sites (Figure 4b). Metastases were most prevalently detected in bone tissue (BMP4 non-granular 19% vs BMP4 granular 28%) (Figure 4b). Nevertheless, no differences were statistically significant, which was likely again due to the low number of events evaluated.

Finally, the potential association between BMP4 expression and patient survival was explored. The overall survival and breast cancer-specific survival did not differ between patients with tumors displaying granular or non-granular BMP4 staining. Since granular BMP4 staining was associated with low proliferation rate, we divided the patients further into subgroups based on the proliferation status but again found no statistically significant associations. Time-to-event analyses were also performed for the time to first metastasis, local recurrence, first bone metastasis and disease-free time. No statistically significant differences were observed for any of the above analyses. In addition, the associations between BMP4 expression and the use of hormone-replacement therapy or BMP7 expression status were also examined, but no differences were found between tumors with granular or non-granular BMP4 staining.

Discussion

BMP4 is a well-known developmental regulator that is widely expressed during early embryogenesis.⁵ However, considerably less is known about BMP4 expression and function in normal adult tissues. Genes and signaling pathways that normally participate in developmental processes have been shown to also be reactivated in cancer pathogenesis.³⁶ Thus, BMP4 and other BMP family members have begun to be a common research topic in the cancer field. Interestingly, BMP2 and BMP7 are currently used in the clinic for healing bone fractures. Although no increase in the risk of cancer has been reported so far, these treatments are not recommended for patients with prior history of cancer.³⁷ In this study, we systematically explored BMP4 protein expression in a comprehensive collection of normal adult and tumor tissues using immunohistochemistry on tissue microarrays. Moreover, a detailed study of BMP4 expression in a large breast cancer patient set was conducted to evaluate the clinical impact of BMP4 expression in breast cancer. It is noteworthy that the antibody used will detect both the mature BMP4 and the more stable precursor protein, and thus the protein expression data do not directly imply the activity of BMP signaling in a given tissue.

BMP4 protein expression was detected in normal and tumor tissues at variable levels. In more than one-fifth of both the benign and malignant samples, BMP4 was strongly expressed in a granular pattern possibly suggesting a presence in secretory vesicles.

BMP4 expression was more often absent in normal tissues compared with tumor tissues, while diffuse expression was more frequent in tumors. BMP4 was also commonly expressed in the epithelial cells and leukocytes within both the normal and tumor samples. Although the number of samples studied for each tissue/tumor type was rather low, the BMP4 expression pattern was generally more uniform among normal samples, whereas tumors commonly showed expression patterns representing all four staining categories.

Our extensive array of normal tissues provided a comparable platform to evaluate BMP4 expression and showed that BMP4 is indeed expressed in a distinct set of adult tissues. Thus, BMP4 is likely involved in the normal physiological functions of certain adult tissues. We found strong granular BMP4 staining in the epithelial cells of the bladder, ureter, uterine cervix and the red pulp area of the spleen for the first time. In the lung epithelium, we observed BMP4 expression patterns similar to previous findings.¹⁵ Strong BMP4 staining was also found in the epithelium of the esophagus and stomach, but a lack of expression was observed in the lower gastrointestinal track (ie, small intestine, colon and rectum). A previous study reported that BMP4 expression was present in inflamed esophageal epithelium and metaplastic Barrett's esophagus but not in normal esophagus.³⁸ Likewise, earlier studies reported little or no BMP4 expression in the stomach; however, *H. pylori* infection was shown to increase BMP4 expression in these tissues.^{28,39} Positive BMP4 expression has been previously reported in the normal ovary and endocrine pancreas.^{12,13} On our tissue microarray, these samples were not optimal for the confirmation of these results as no follicles were present in the ovary sections and the endocrine cells were not well represented in the pancreas, which possibly explains the lack of BMP4 expression in these tissues within our sample set. Although direct comparisons between mRNA and protein expression data are not straightforward, public microarray data also demonstrate high *BMP4* expression in the urogenital system, gastrointestinal organs and uterine cervix (www.genesapiens.org), thus further corroborating our findings.

The main advantage of our data compared with previous studies is that the tissue microarray format permits the identical handling of all samples, and the scoring of the data from different tissues can be performed according to comparable criteria. Notably, the previous BMP4 expression data from normal adult tissues often stemmed from studies of cancer or other diseases where the normal tissue samples were obtained as paired samples in close proximity of tumor or diseased tissue. This naturally raises some concerns regarding the 'normalness' of the samples. Nevertheless, we fully agree that the number of parallel samples in our study was low; however, their uniform staining patterns still highlights the usefulness of our data.

In cancer samples, strong BMP4 expression was observed in the squamous cell carcinomas. We demonstrate for the first time strong, granular BMP4 expression in the squamous cell carcinomas of the uterine cervix, skin and lung. Our results from head and neck and esophageal squamous cell carcinomas agree with previous findings.^{30,40,41} In addition to these results, the immunohistochemical analysis of BMP4 protein expression in tumor samples has only been performed in colorectal, ovarian, gastric, renal and hepatocellular carcinomas as well as in a few samples of melanoma. In colorectal and gastric cancers, BMP4 expression had been identified in the majority of the samples, but strong expression was detected only in a minority, which resembled the staining pattern we also detected.^{20,28} Our results are also in agreement with previous findings regarding the BMP4 expression in ovarian cancer;²⁹ however, in melanoma and renal and hepatocellular carcinomas, strong BMP4 staining has been previously detected more often than our data indicated.^{19,27,42}

In this study, we also show that tumor types originating from the same organ or tissue, such as those from lung or skin, can drastically differ in terms of their BMP4 expression. According to earlier reports, BMP4 protein expression was detected in a minority of a small panel of non-small cell lung carcinomas, but studies on the mRNA level of *BMP4* did not indicate any differences between the subtypes of lung cancer.^{43,44} In the case of skin, no tumor subtypes besides melanoma had been previously examined.¹⁹ In the tumor tissue microarray, low levels of BMP4 protein expression were detected in cancers of the prostate and brain that have only been shown to express *BMP4* at the mRNA level.^{45,46} For malignancies originating from the thyroid, uterus, spleen and lymph nodes, this is the first study to examine BMP4 protein expression in these tissues.

Similarly to the normal tissues, comparison of our tumor protein expression data and the public mRNA expression data show a good concordance. High *BMP4* mRNA expression has been commonly detected for example in lung, gastrointestinal, pancreatic, ovarian and uterine cancers whereas lower expression was seen in prostate carcinomas (www.genesapiens.org). However as stated above, such comparisons are not unproblematic and especially in the case of tumor samples, one may expect more variability in both mRNA and protein levels between different samples.

Finally, the BMP4 expression patterns in the tumor samples were compared with the patterns detected in the corresponding normal tissues. In four organs where BMP4 expression was strong in the normal tissue (ie, stomach, esophagus, liver and spleen), only a subset of the tumor tissue samples expressed BMP4 at a similar level, which implies the expression of BMP4 is reduced in subset of cancers. However, in a previous comparison of

adjacent normal and tumor tissues from the stomach, liver and esophagus, BMP4 expression was higher in the tumor samples.^{28,40,47} Notably, our normal and tumor tissue microarrays were truly independent samples and not paired unlike previous studies. In the case of four normal tissues (ie, colon, pancreas, thyroid and ovaries) where no BMP4 expression was detected, a subset of the corresponding tumor samples had strong granular BMP4 staining indicating increased BMP4 expression. In colorectal cancer, a similar trend of increased BMP4 expression in tumor tissue has also been observed.²⁰ In the prostate and uterine cervix, no differences between the normal and cancer samples were observed, but in the case of brain, lung and skin tissues, the interpretation was not so clear. BMP4 expression in the normal brain tissue was possibly present in the microglial cells, whereas in the tumor tissue, a similar pattern was not evident. Additionally, in the lung, the majority of the tumor samples were similarly granularly stained compared with normal lung with the exception of small cell carcinoma.

For breast cancer, this study is the first report to examine BMP4 protein expression with comprehensive patient material. We have previously shown that *BMP4* mRNA is expressed at variable levels in a small panel of primary breast tumors.^{23,33} Similarly to our previous observations, the majority of patient samples also displayed a relatively low BMP4 protein expression level. In 23% of the samples, BMP4 was expressed in a strong granular manner. The frequency of strong BMP4 expression in breast cancer was similar to the overall frequency observed in other tumor types. In the normal mammary gland tissue, BMP4 was detected only at a low level implying that strong expression is cancer specific. In our previous study, *BMP4* mRNA expression was observed more often in low-grade tumors,²³ but this observation did not translate to the protein level. However, strong BMP4 expression was more common in invasive lobular carcinomas than in ductal carcinomas. We have observed a similar expression pattern for BMP7 in the same breast cancer patient set.³⁵ Interestingly, strong BMP4 expression was also observed more often in slowly proliferating tumors, and at the same time, strong BMP4 expression correlated with an increased tumor recurrence. In our previous *in-vitro* functional studies, BMP4 expression decreased breast cancer cell growth and at the same time was able to increase the migration and invasion of these same cells.²³ Similarly, BMP4 expression induced an invasive phenotype in a 3D model of normal mammary epithelial cells and reduced their proliferation in concert with other growth factors.^{48,49} Thus, our current data in breast cancer patients confirm these *in-vitro* observations and suggest that BMP4 is indeed a bidirectional player that harbors both tumor-suppressive and tumor-promoting features in breast cancer. Previously, a low BMP4 methylation score, together with other methylation markers, correlated with a longer time to distant metastasis in breast

cancer⁵⁰; however, we did not observe any significant changes in our time-to-event analyses. Overall, the involvement of BMP4 in cancer progression has been studied on a very limited scale. In squamous cell carcinomas of the head and neck, strong BMP4 expression was detected particularly in tumor samples of patients with metastases and was associated with a shorter overall survival.³⁰ On the contrary, in ovarian cancer, strong BMP4 expression associated with a longer progression-free time, and in gastric cancer, BMP4 was detected more often in the non-metastatic tumors.^{28,29} Interestingly in the ovarian cancer study, the authors also showed that *BMP4* expression is induced by cisplatin treatment but that only the pretreatment level was associated with patient outcome.²⁹ These findings emphasize the notion that BMP4's role is most likely highly diverse in different cancer types.

In conclusion, we have shown that BMP4 is expressed in a strong, granular manner in several normal tissues, which indicates that it is likely to have important functions in normal tissue homeostasis. Variable patterns of expression were detected in multiple tumor samples with particularly strong BMP4 staining observed in squamous cell carcinomas. Most importantly, in breast cancer, strong BMP4 expression associated with low proliferation index of the tumors and an increase in tumor recurrence. These observations concur with our previous *in-vitro* cell line data illustrating a dualistic function for BMP4 as a growth inhibitor and promoter of migration and invasion in breast cancer. A similar dualistic role has been previously established for the superfamily member TGF β , which functions as a tumor suppressor during the first steps of tumor formation, but it is later found to stimulate cancer metastasis.^{51,52}

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (<http://www.nature.com/modpathol>)