Breast Cancer Research and Treatment (2019) 174:365–373
https://doi.org/10.1007/s10549-018-5067-5

PRECLINICAL STUDY

Genomic profile of breast sarcomas: a comparison with malignant phyllodes tumours

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Received: 8 October 2018 / Accepted: 20 November 2018 / Published online: 3 December 2018

Abstract

Purpose We aimed to investigate the genomic profile of breast sarcomas (BS) and compare with that of malignant phyllodes tumours (MPT).

Methods DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) specimens from 17 cases of BS diagnosed at Singapore General Hospital from January 1991 to December 2014. Targeted deep sequencing and copy number variation (CNV) analysis on 16 genes, which included recurrently mutated genes in phyllodes tumours and genes associated with breast cancer, were performed on these samples. Genetic alterations (GA) observed were summarised and analysed.

Results Nine cases met the quality control requirements for both targeted deep sequencing and CNV analysis. Three (33.33%) were angiosarcomas and 6 (66.67%) were non-angiosarcomas. In the non-angiosarcoma group, 83.33% ($n=5$) of the patients had GA in the *TERT* gene. The other commonly mutated genes in this group of tumours were *MED12* ($n=4$, 66.67%), *BCOR* ($n=4$, 66.67%), *KMT2D* ($n=3$, 50%), *FLNA* ($n=3$, 50%) and *NF1* ($n=3$, 50%). In contrast, none of the angiosarcomas had mutations or copy number alterations in *TERT*, *MED12*, *BCOR*, *FLNA* or *NF1*. Eighty percent of patients with GA in *TERT* ($n=5$) had concurrent mutations in *MED12*. Sixty percent ($n=3$) of these cases also demonstrated GA in *NF1*, *PIK3CA* or *EGFR* which are known cancer driver genes.

Conclusions The non-angiosarcoma group of BS was found to share similar GA as those described for MPT, which may suggest a common origin and support their consideration as a similar group of tumours with regard to management and prognostication.

Keywords Mesenchymal · Genomic profile · *TERT* · *MED12* · *NF1*

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Introduction

Breast sarcoma (BS) is an uncommon malignant mesenchymal mammary neoplasm which comprises a diverse array of histological subtypes originating from various cell lineages such as fibrous, adipose, endothelial and muscle tissues. It accounts for less than 1% of all malignant breast tumours and less than 5% of all sarcomas [1, 2]. It has an aggressive nature with high risk of recurrence and poor prognosis. Based on our previous study, the 5-year disease-free survival in BS patients is 59.1%, consistent with findings in the literature [3–5]. Angiosarcoma appeared to be a distinct subtype with a significantly poorer outcome [3]. Most studies on this subject focused on describing the clinicopathological features and survival outcomes of BS based on individual reports or small case series [1, 4–15]. Though *p53*, *RB*, *PI3K* and *IDH* gene mutations have been identified as the most prevalent mutations in sarcomas, there is limited information on the genomic profile unique to BS [16].

Due to similarities in their histological appearance, BS has often been compared to malignant phyllodes tumours (MPT). MPT form 10 to 20% of all phyllodes tumours [17]. Phyllodes tumour is a biphasic fibroepithelial neoplasm of the breast, with typical features of leafy stromal fronds capped by bilayered epithelium. In MPT, the stromal tissue often shows a sarcomatous pattern while the epithelial component is benign. Different from benign and borderline phyllodes tumours, it is believed that stromal proliferation in MPT is autonomous and no longer requires a mitogenic stimulus from the epithelium [18–22]. In recent years, there have been significant discoveries in the genomic landscape of MPT. Based on a study from Japan, Yoshida et al observed that *TERT* promoter mutations were common and often associated with *MED12* mutations in phyllodes tumours [23]. Through microdissection-based analysis, they discovered that *TERT* promoter mutations were present only in the stromal tissue and not in the epithelial component. This further supported the notion that stromal cells formed the principal neoplastic component in phyllodes tumours. In a study involving 100 fibroepithelial tumours, Tan et al found frequent mutations in *MED12* and *RARA* genes [24]. They further described that in borderline and malignant phyllodes tumours, there were additional mutations in cancer driver genes such as *NF1*, *PIK3CA* and *EGFR* which are known for their transforming ability. The above findings were demonstrated again in a more recent work by Nozad et al who performed a comprehensive genomic profiling of MPT. They demonstrated that the commonly mutated genes in MPT included *TERT* promoter (57.9%), NF1 (45.8%) and MED12 (45.8%) [25].

Echoing findings from previous studies in the literature, we analysed 62 cases of BS and MPT in our institution and observed that they shared similar clinicopathological features and survival outcomes [2, 3, 26–29]. In order to further interrogate the relationship between MPT and BS, this study aimed to investigate the genomic profile of BS and demonstrate if a similar mutation pattern exists between these two groups of tumours.

Materials and methods

Patients and tumours

Cases of BS diagnosed at the Department of Pathology, Singapore General Hospital from January 1991 to December 2014 were derived from the department database. This study was approved by the institutional review board (CIRB Ref: 2015/2697 and 2005/002/F). Patients with metastatic disease at presentation were excluded from the study. Histopathological slides for each case were reviewed by a single pathologist to confirm the diagnosis of BS and classify them into different subtypes such as undifferentiated pleomorphic sarcoma, angiosarcoma and osteosarcoma (Fig. 1). Undifferentiated pleomorphic sarcoma is a diagnosis of exclusion. Our approach in making this diagnosis was premised on morphologically malignant pleomorphic and giant tumour cells in addition to spindle cells (which may predominate) and epithelioid cells. While it may have overlapping histological features with other mesenchymal tumours including pleomorphic variants of malignant peripheral nerve sheath tumour (MPNST), leiomyosarcoma and rhabdomyosarcoma, immunohistochemical studies did not demonstrate any lineage-specific markers including S100, SOX10, MyoD1, myogenin, CD31. Undifferentiated pleomorphic sarcoma may be variably positive for smooth muscle actin and CD34. Vimentin was not helpful as it decorates many non-mesenchymal malignancies in addition to sarcomas. Focal cytokeratin expression may be discovered in a very small percentage of these tumours. We diagnosed osteosarcoma based on identifying osteoid formation by malignant cells. In our view, no immunohistochemical studies are specifically helpful, so extensive sampling and detailed morphological examination are needed. For angiosarcoma, the diagnosis was made based on the identification of a vasoformative tumour with anastomosing channels permeating into breast parenchyma, invading through and effacing breast lobules, accompanied by variable endothelial atypia. During the review of the slides, tumour areas were identified and micro-dissected to facilitate the subsequent DNA extraction process. Genomic DNA from formalin-fixed, paraffin-embedded (FFPE) slides was then extracted and purified using the Qiagen GeneRead DNA FFPE tissue kit (Qiagen) following the manufacturer's protocol. The genomic DNA yield and quality were determined using a Nanodrop ND1000 spectrophotometer

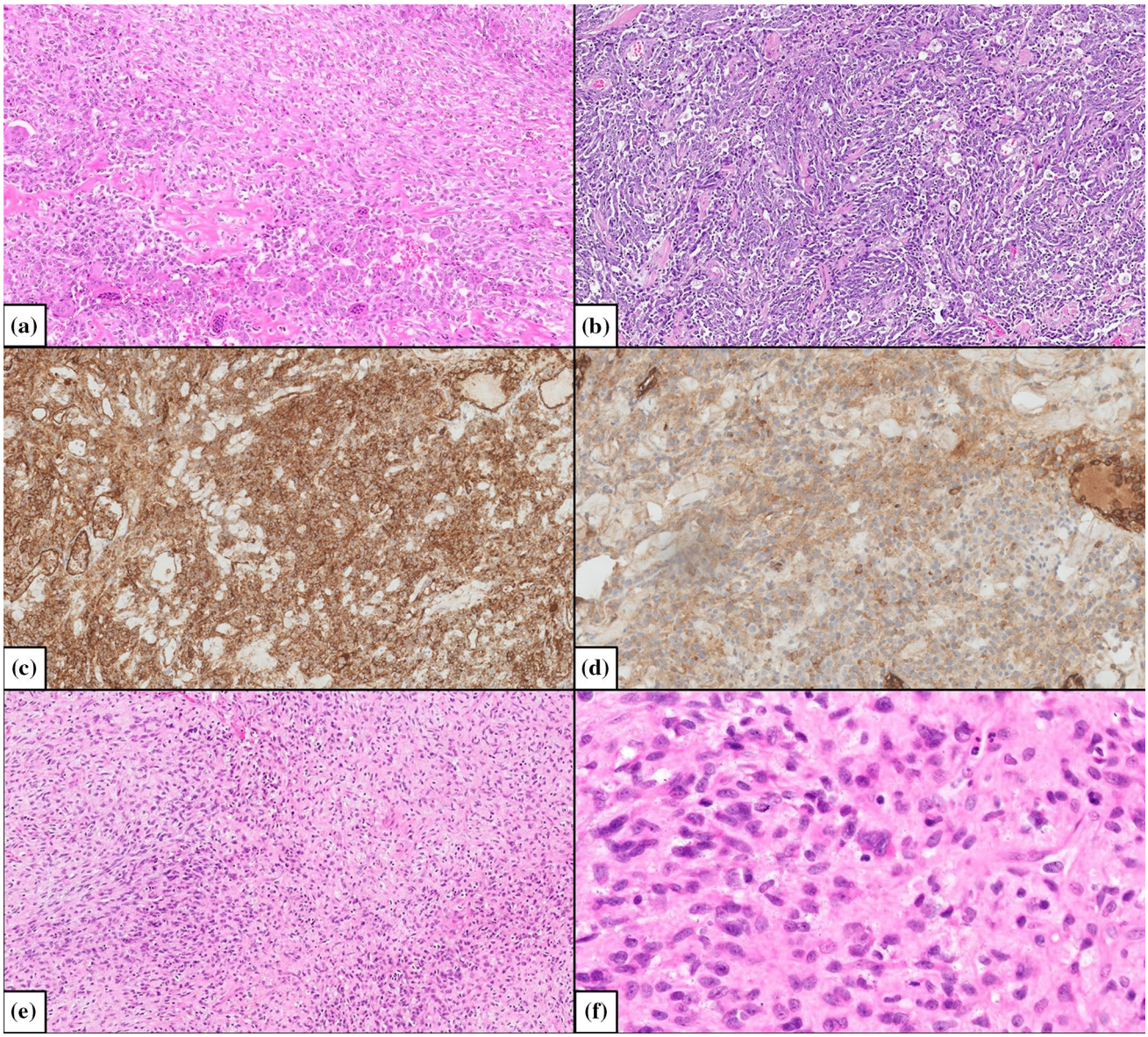

Fig. 1 Images of **a** osteosarcoma **b** angiosarcoma **c** immunohistochemical expression of CD31 in angiosarcoma and **d** immunohistochemical expression of F8 in angiosarcoma **e** undifferentiated pleomorphic sarcoma (low magnification) **f** undifferentiated pleomorphic sarcoma (higher magnification)

(Thermo Fisher) and PicoGreen® dsDNA quantitation assay (Thermo Fisher).

Targeted deep sequencing

Libraries were prepared according to the manufacturer's protocol for 10 samples that had a minimum of 50 ng of gDNA, with a QIAseq™ Targeted Custom DNA Panel (Qiagen). The panel consisted of 16 genes which included recurrently mutated genes in phyllodes tumours identified in our previous study and genes associated with breast cancer (Fig. 2) [24]. The enriched libraries were sequenced on Illumina HiSeq 2500 platform to generate 150 bp paired-end reads.

Quality of the FASTQ files was assessed with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The first 30 bp of sequencing reads were trimmed to remove adapters using fastx_trimmer (http://hannonlab.cshl.edu/fastx_toolkit/). Trimmed paired reads were mapped to hg19 (hs37d5) using BWA-MEM (v0.7.15-r1140) and sorted or indexed with SAMtools [30–32]. Variants were called using FreeBayes (v1.1.0-4-gb6041c6, settings: -m 30 -q 30 -F 0.01 -u) and annotated with wANNOVAR [33, 34]. Variants were filtered to retain only those covered by at least 100

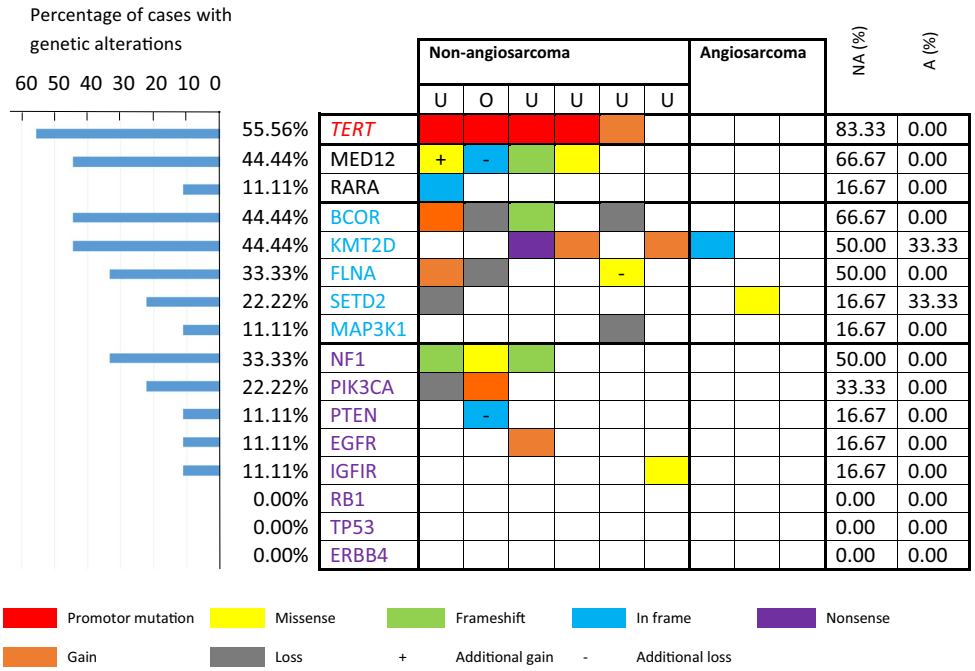

Fig. 2 Genomic profile of breast sarcoma. Layout of genetic alterations (GA) identified by targeted deep sequencing and copy number variation analysis in 9 breast sarcomas. Top, the histological groups are indicated as non-angiosarcoma (NA) and angiosarcoma (A). The non-angiosarcoma cases are further labelled as undifferentiated pleomorphic sarcoma (U) and osteosarcoma (O). Centre, 16 genes including recurrently mutated genes in phyllodes tumours identified in our previous study and genes associated with breast cancer. They are categorised by colours: red, *TERT* gene; black, genes associated with all fibroepithelial tumours; blue, genes associated with phyllodes tumours; purple, cancer driver genes. Coloured rectangles indicate the GA categories seen in the cases (somatic mutations and copy number variations). + and − signs denote additional copy number alterations in the same patient. Left, the percentage of cases with GA in each gene. Right, frequency of GA by histological subtype

reads and having at least 5 variant reads. In addition, the cut offs for variant allele frequencies (VAFs) and indels were set at less than 5%. Annotated variants were manually curated using Integrative Genomics Viewer (IGV) to exclude likely sequencing artefacts [35].

Copy number variation (CNV) analysis

Copy number estimates for each of the genes included in the targeted sequencing study were obtained using OncoScan [36]. The DNA samples which passed the quality control check were put through annealing with molecular inversion probes (MIP) according to the Affymetrix recommendations. Each MIP had two homology regions for hybridisation to a specific DNA target region with a gap in between them. Directions of the homology regions were designed to generate an incomplete circular form between the DNA target and the probe. Annealed products were then gap-filled with Adenine and Thymine or Guanine and Cytosine by DNA polymerase. All uncircularised probes and DNA templates were then digested by exonucleases. The gap-filled circular probes were cleaved to generate linear probes and put through amplification with polymerase chain reaction using Taq Polymerase. Fragmented products were hybridised to OncoScan CNV arrays (Affymetrix). Arrays were washed and stained in the Affymetrix fluidics station using the OncoScan CNV protocol. They were then scanned with an Affymetrix 3000 7G scanner. Array fluorescence intensity files were generated and used for data analysis. CNVs were detected by dividing the normalised signal intensity of the specimens by that of the reference data. OncoScan uses a reference data set from 400 normal FFPE samples of various sources including different geographic locations, gender, age and tissue types [37]. The computation was displayed as the base 2 logarithm of the quotient of the division, thus a negative value reflected a copy number loss while a positive value reflected a copy number gain.

Statistical analysis

Genetic alterations (GA) included all somatic mutations and copy number variations. The findings were analysed using SPSS software, Version 21 (SPSS, Inc., Chicago, IL, USA). Fisher's exact test was used to evaluate the differences in the frequency of GA between the different groups. An alpha-level of 0.05 was considered as statistically significant.

Results

Based on our previous study, we identified 17 cases of BS diagnosed in the Department of Pathology, Singapore General Hospital from January 1991 to December 2014. DNA was extracted from FFPE specimens of all the 17 cases of BS. Nine cases met the quality control requirements for both targeted deep sequencing and CNV analysis. All patients were female and the median age was 54 years (range 42–74). The samples used for DNA analysis were all obtained from the breast specimens. Among the 9 cases, 3 (33.33%) were angiosarcomas, while 6 (66.67%) were non-angiosarcomas (5 undifferentiated pleomorphic sarcomas and 1 osteosarcoma). The clinicopathological features are described in Table 1.

There was a total of 36 GA identified in 16 genes within the 9 BS. The median number of GA per case was 3 (range 0–9 GA per case). All except 1 case had GA in at least 1 gene ($n=8$, 88.89%). In general, the most commonly mutated genes were *TERT* ($n=5$, 55.56%), *MED12* ($n=4$, 44.44%), *BCOR* ($n=4$, 44.44%) and *KMT2D* ($n=4$, 44.44%) (Fig. 2).

In the non-angiosarcoma group, 83.33% ($n=5$) of tumours possessed *TERT* promoter mutations ($n=4$) or copy number alteration ($n=1$). The nucleotide change in all cases with *TERT* promoter mutations were − 124C > T. The other commonly mutated genes in this group of tumours were *MED12* ($n=4$, 66.67%), *BCOR* ($n=4$, 66.67%), *KMT2D* ($n=3$, 50%), *FLNA* ($n=3$, 50%) and *NF1* ($n=3$, 50%). All mutations in the *MED12* gene occurred in exon 2. In contrast, none of the angiosarcomas had mutations or copy

Table 1 Clinicopathological features of breast sarcomas

Clinicopathological parameter	Non-angiosarcoma ($n=6$)		Angiosarcoma ($n=3$)		Total ($n=9$)	
Age (years)	Mean	59.00	Mean	52.00	Mean	56.67
	Median	56.50	Median	44.00	Median	54.00
	Range	50–74	Range	42–70	Range	42–74
Ethnicity						
Chinese	6 (100.00%)		2 (66.67%)		8 (88.89%)	
Indian	0 (0.00%)		1 (33.33%)		1 (11.11%)	
Family history of breast cancer						
Yes	2 (33.33%)		0 (0.00%)		2 (22.22%)	
No	4 (66.67%)		3 (100.00%)		7 (77.78%)	
Personal history of previous radiation to chest						
Yes	1 (16.67%)		1 (33.33%)		2 (22.22%)	
No	5 (83.33%)		2 (66.67%)		7 (77.78%)	
Menopausal status						
Premenopausal	1 (16.67%)		2 (66.67%)		3 (33.33%)	
Postmenopausal	5 (83.33%)		1 (33.33%)		6 (66.67%)	
Presentation						
Mass	6 (100.00%)		3 (100.00%)		9 (100.00%)	
Others	0 (0.00%)		0 (0.00%)		0 (0.00%)	
Histology						
Undifferentiated pleomorphic sarcoma	5 (83.33%)		0 (0.00%)		5 (55.56%)	
Osteosarcoma	1 (16.67%)		0 (0.00%)		1 (11.11%)	
Angiosarcoma	0 (0.00%)		3 (100.00%)		3 (33.33%)	
Size (mm)	Mean	117.67	Mean	110.67	Mean	115.33
	Median	95.00	Median	55.00	Median	80.00
	Range	20–250	Range	47–230	Range	20–250
Multifocality						
Yes	1 (16.67%)		0 (0.00%)		1 (11.11%)	
No	5 (83.33%)		3 (100.00%)		8 (88.89%)	
Grade						
Low	1 (16.67%)		0 (0.00%)		1 (11.11%)	
Intermediate	0 (0.00%)		0 (0.00%)		0 (0.00%)	
High	5 (83.33%)		3 (100.00%)		8 (88.89%)	

number alterations in *TERT*, *MED12*, *BCOR*, *FLNA* or *NF1* (Table 2).

Notably 80% of patients with GA in *TERT* ($n=5$) had concurrent mutations in *MED12*. Additionally, 60% ($n=3$) of these cases also disclosed GA in *NF1*, *PIK3CA* or *EGFR* which were known as cancer driver genes with transforming ability (Fig. 2).

Discussion

Our current study showed that *TERT* promoter mutations and copy number alterations were frequently identified in the non-angiosarcoma group of BS. The majority of these cases also had concurrent mutations in *MED12*. To date, mutations in the *TERT* promoter and *MED12* gene are the most frequent GA reported in phyllodes tumours [23–25]. Yoshida et al showed in their cohort that 65% of phyllodes tumours had *TERT* promoter mutations. Subset analysis revealed that 62% of MPT possessed *TERT* promoter mutations. All except one case had concurrent mutations in MED12, indicating the strong association between these two groups of mutations. Thus they may have a synergistic relationship in the tumourigenesis of phyllodes tumours. More importantly, they observed that these mutations only occurred in the stromal component of the tumours and further supported the idea of stromal cells being the sole neoplastic component in fibroepithelial lesions [23]. More recently, Nozad et al shared their findings on the comprehensive genomic profiling of MPT. They again demonstrated that the most commonly mutated genes in MPT included *TERT* promoter (57.9%) and *MED12* (45.8%) [25]. Notably, all 11 *TERT* promoter mutations in their MPT group were − 124 C > T, which was the same mutation found in our non-angiosarcoma group.

In this present study, we observed that in the non-angiosarcoma group of BS, mutations and copy number variations also commonly occurred in the *BCOR*, *KMT2D* and *FLNA* genes. GA in *NF1*, *PIK3CA* or *EGFR* which were the known cancer driver genes occurred in more than half of the BS cases with GA in *TERT* gene. Mutations in these cancer driver genes may play a crucial role in the ultimate malignant change of these tumours. A similar mutation pattern was observed in MPT. In 2015, Tan et al published their results on the genomic landscapes of breast fibroepithelial tumours. They observed that while *MED12* and *RARA* gene mutations were common to all fibroepithelial tumours, mutations in *BCOR*, *KMT2D* and *FLNA* were seen mainly in phyllodes tumours and rarely in fibroadenoma. Above all, MPT particularly exhibited additional GA in *NF1*, *PIK3CA* and *EGFR* [24]. The above findings highlight the similarities in the genomic profile between MPT and the non-angiosarcoma group of BS.

Based on our previous study, we found that patients with breast angiosarcomas had a much poorer disease-free survival outcome as compared to other BS and MPT [3]. This concurred with the findings in several other studies [4, 38]. Among BS, Zelek et al. found that angiosarcoma

Table 2 Comparison of genetic alterations between non-angiosarcoma and angiosarcoma groups

Genetic alterations	Tumour groups		*P* value (95% confidence interval)
	Non-angiosarcoma	Angiosarcoma	
TERT			
Yes	5 (83.33%)	0 (0.00%)	0.048 (0.028–0.997)*
No	1 (16.67%)	3 (100.00%)	
MED12			
Yes	4 (66.67%)	0 (0.00%)	0.167 (0.108–1.034)
No	2 (33.33%)	3 (100.00%)	
BCOR			
Yes	4 (66.67%)	0 (0.00%)	0.167 (0.108–1.034)
No	2 (33.33%)	3 (100.00%)	
FLNA			
Yes	3 (50.00%)	0 (0.00%)	0.464 (0.225–1.113)
No	3 (50.00%)	3 (100.00%)	
NF1			
Yes	3 (50.00%)	0 (0.00%)	0.464 (0.225–1.113)
No	3 (50.00%)	3 (100.00%)	
KMT2D			
Yes	3 (50.00%)	1 (33.33%)	1.000 (0.242–2.325)
No	3 (50.00%)	2 (66.67%)	

*Statistically significant

was the only histologic subtype significantly associated with an unfavourable outcome. In their series, the 10-year disease-free and overall survivals for patients with angiosarcomas were as low as 0% [38]. A separate group of French investigators also reported that patients with angiosarcomas had the worst prognosis. The 5-year disease-free survival was only 27% [4]. All these findings showed that angiosarcoma is not only histologically distinct from other BS, but also inherently more aggressive in their disease behaviour. Thus it was not surprising to observe a clear difference in the genomic profile between these two groups. In our present study, none of the angiosarcomas had GA in *TERT*, *MED12*, *BCOR*, *FLNA* or *NF1*. Unfortunately, the key genetic and molecular aberrations associated with the tumourigenesis of angiosarcomas remain unclear. To date, the most commonly reported GA in angiosarcomas occur in the *MYC* gene [39–41]. In 2012, Italiano et al found *MYC* amplification in 50% of primary angiosarcomas [41]. More recently, Fraga-guedes et al also observed MYC amplification in 54% of secondary breast angiosarcomas [39]. The other genes known to be altered in angiosarcomas include *FLT4*, *MAML1*, *BRCA1* and *2* genes [41–43].

We have shown that the non-angiosarcoma group of BS are similar to MPT not only in their clinicopathological features and survival outcomes, but also in their genomic profile. Despite the limitation of small sample numbers, we believe that our findings support the concept that some BS originated from MPT, where the diagnostic epithelial component is no longer present due to overgrowth of the neoplastic stromal component. Thus it is reasonable to regard these tumours as a single group for management and prognostication.

Conclusion

BS and MPT are rare conditions with great heterogeneity. The non-angiosarcoma group of BS was found to share similar GA as compared to MPT. Thus, these tumours could be considered as a group of diseases for management and prognostication.

Acknowledgements This work was supported by funding from the Singapore General Hospital Research Grant 2016.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the institutional research board.

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