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Identification of Genes Associated with Local Aggressiveness and Metastatic Behavior in Soft Tissue Tumors[1,2]

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Abstract

Soft tissue tumors represent a group of neoplasia with different histologic and biological presentations varying from benign, locally confined to very aggressive and metastatic tumors. The molecular mechanisms responsible for such differences are still unknown. The understanding of these molecular alterations mechanism will be critical to discriminate patients who need systemic treatment from those that can be treated only locally and could also guide the development of new drugs' against this tumors. Using 102 tumor samples representing a large spectrum of these tumors, we performed expression profiling and defined differentially expression genes that are likely to be involved in tumors that are locally aggressive and in tumors with metastatic potential. We described a set of 12 genes (*SNRPD3*, *MEGF9*, *SPTAN-1*, *AFAP1L2*, *ENDOD1*, *SERPIN5*, *ZWINTAS*, *TOP2A*, *UBE2C*, *ABCF1*, *MCM2*, and *ARL6IP5*) showing opposite expression when these two conditions were compared. These genes are mainly related to cell-cell and cell–extracellular matrix interactions and cell proliferation and might represent helpful tools for a more precise classification and diagnosis as well as potential drug targets.

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Introduction

Soft tissue tumors are a heterogeneous group of mesenchymal tumors with diverse histologic presentation and clinical behavior [1]. Histologically, soft tissue tumors are classified in more than 50 subtypes, based on their cellular differentiation and morphologic findings. In this study, peripheral neural tumors were also included because their morphology, clinical behavior, and treatment are similar to soft tissue mesenchymal tumors and they are also considered soft tissue tumors.

According to their biological behavior, they can be grouped into three major categories, benign mesenchymal tumors (BMTs), tumors with local aggressiveness but with no metastatic potential, and sarcomas (malignant mesenchymal tumors [MMTs]) that have both local aggressiveness and metastatic potential. The latter group can be further subdivided as low-, intermediate-, or high-grade tumors according to classifications of the National Cancer Institute and Fédération Nationale des Centres de Lutte Contre le Cancer. The National Cancer Institute system uses a combination of histologic type, cellularity, pleomorphism, and mitotic rate [2]. The Fédération Nationale des Centres de Lutte Contre le Cancer system is based on a score by evaluating three parameters, namely tumor differentiation, mitotic rate, and amount of necrosis. The score is attributed independently to each parameter, and the grade is a result of its adding [3].

At the molecular level, sarcomas can be characterized by the presence or the absence of tumor-specific mutations. For instance, alveolar rhabdomyosarcomas are characterized by t(1;13) (*PAX7;FKHR*) or t(2;13) (*PAX3;FKHR*) translocations, whereas synovial sarcomas have specific t(X;18) (*SSX;SYT*) translocation. In contrast, leiomyosarcomas and pleomorphic sarcomas lack specific chromosome alterations [4].

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[2]This article refers to supplementary materials, which are designated by Figure W1 and Tables W1 to W3 and are available online at www.transonc.com. Supplementary data are also available at http://www.maiges.org/sarcomaFibromatosis/.
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Sarcomas represent approximately 1% of adult malignancies but, despite this low incidence, are often of poor prognosis, at a discrepancy with their benign counterpart such as schwannomas, lipomas, and leiomyomas that are usually well-circumscribed tumors, with no local aggressiveness and without metastatic potential. In between these two extremes, there are some subtypes of mesenchymal tumors that have characteristics of both groups. They are locally aggressive but lack metastatic potential. One classic example is desmoids tumors, also known as desmoid-type fibromatosis (DTF). They are clonal tumors, with fibroblastic proliferation and local aggressiveness but without metastatic potential. They occur with higher frequency in chest and abdominal wall, thigh, and head and neck region. Local recurrence is frequent, and they can be fatal owing to local effects, especially in the head and neck region [5].

Whereas tumor size and histologic features are the best prognostic factors available for mesenchymal tumors, little is known about molecular alterations that could contribute to the understanding of cell origin, malignant transformation, and tumor biology. Also, few molecular markers were identified as having diagnostic and prognostic values.

Gastrointestinal stromal tumors (GISTs) are one of the few successful examples of mesenchymal tumors in which the molecular events related to malignant transformation are well established. These tumors usually have an activating mutation of *C-Kit* gene and can be treated with imatinib mesylate, a tyrosine kinase inhibitor [6]. Another example of a gene that was recently described as a sensitive and specific immunohistochemical marker for synovial sarcoma is TLE1 [7]. TLE proteins are transcriptional corepressors that inhibit Wnt signaling and have a role in repressing differentiation. Measurement of TLE1 expression might have applications for diagnosis and eventually for the understanding of tumor biology.

Several others studies using microarray technology had been reported mainly to describe gene expression signature associated with histologic differentiation or outcome in specific histologic subtypes [8–16].

It is clear that sarcomas have distinct pathways related to malignant transformation and cellular differentiation and some of molecular alterations can be pinpointed to specific chromosomal translocations that are pathognomonic for specific tumors. Nevertheless, tumors arising from distinct pathways and/or cell types can be grouped as a function of their biological behavior and defined by their histologic grade of malignancy. Importantly, tumors with similar behavior are treated similarly. Furthermore, most sarcomas have metastatic dissemination through blood vessels with exception for those with epithelial differentiation that can also have lymphatic dissemination. Because our goal is the understanding of biological behavior, rather than sarcogenesis, we grouped samples regardless of histologic classification, favoring metastatic potential. In an effort to identify genes that could be implicated in aggressiveness and/or metastatic behavior of sarcomas, we compared the expression profile of a set of 102 samples representing benign soft tissue tumors, DTF, and sarcomas. Here, we describe a set of altered genes that, on the basis of their function, are candidates for playing a role in the biology of soft tissue tumors and, hence, are potential drug targets. These genes might also serve as prognostic factors.

Materials and Methods

Patients and Samples

Patients were recruited at Hospital do Cancer AC Camargo (São Paulo/Brazil) during an 8-year period (1997-2004). All patients signed a preinformed consent and the study was approved by our internal review board (664/04). Tissue samples were provided by the AC Camargo Hospital Tumor Bank. Tissue samples obtained by surgery were snap frozen in liquid nitrogen, whereas biopsy samples were collected in RNAlater (Ambion, Austin, TX). All samples were then stored at −140°C until further processing. At the time of RNA extraction, diagnosis was reconfirmed by hematoxylin and eosin staining. Frozen samples were hand-dissected for removal of infiltrating inflammatory cells and for enrichment of tumor. For proper tumor classification, immunohistochemistry using a panel of antibodies was done in the corresponding paraffin-formalin–embedded blocks. A detailed description of the 102 samples is presented in Table W1. For immunohistochemistry, we evaluated a total of 253 cases, including 101 fibromatosis, 38 synovial sarcomas, 37 leiomyosarcomas, 33 pleomorphic sarcomas, 10 fibrosarcomas, 9 liposarcomas, 7 malignant peripheral nerve sheet tumors, 6 GISTs, 6 neurofibromas, 4 alveolar soft part sarcomas, 4 leiomyomas, and 4 schwannomas, retrieved from archived samples.

Extraction, Amplification, and Labeling of the Amplified RNA

Total RNA was extracted using Trizol (Life Technologies, Inc, Grand Island, NY) and amplified by a T7-based protocol [17]. As described by Pollack [18], all samples were compared with a reference RNA. We used a pool of RNA representing equal total RNA concentration for 15 human cell lines. For replica hybridizations with dye-swap, amplified RNA (3 mg) was added to synthetic antisense RNA corresponding to internal controls, and labeled indirectly, with either Alexa Fluor 555 or Alexa Fluor 647 (catalog no. A32757; Molecular Probes, Carlsbad, CA).

Hybridization and Scanning of Complementary DNA Microarray

Glass arrays containing 4800 spots, of which 4566 are unique complementary DNA (cDNA) sequences, were prepared in our laboratory with the aid of the Flexys robot (Genomic Solutions, UK [23]). Detailed descriptions are available at Gene Expression Omnibus data repository under accession number GPL1930, and the accession number for raw data is GSE14541 (http://www.ncbi.nlm.nih.gov/projects/geo).

Prehybridization, hybridization, and washing were performed as previously described [17] and slides were scanned on a confocal laser scanner (ScanArray Express; PerkinElmer Life Sciences, Waltham, MA). Data were extracted with ScanArray Express software (PerkinElmer Life Sciences) using the histogram method.

Quantitative Polymerase Chain Reaction

For validation of array data, we used 27 samples also used in array analysis (5 BMTs, 6 DTFs, and 16 MMTs) plus 14 independent samples (12 BMTs and 2 DTFs). A detailed description of samples is presented in Table W1.

Aliquots of 2 μg of total RNA were reverse-transcribed in the presence of 500 ng of oligo(dT15) in a final reaction volume of 20 μl using Impron II Reverse Transcriptase System (Promega, Madison, WI). Primer pairs for real-time polymerase chain reaction (PCR) were determined with the aid of Primer Express software 3.0 (Applied Biosciences, Carlsbad, CA) using default parameters. The primer sets are described in Table W2. Reactions were performed in the presence of 10 ng of cDNA product using SYBR Green Master Mix system according to the manufacturer's instructions. Reactions were performed in duplicate on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Carlsbad, CA) and analyzed with Sequence Detection Software (version 2.3). The C_t values were transformed into units using comparative C_t

method [19], and the normalization factor for each sample was calculated using Genorm software based on the expression level of endogenous HMBS, BCR2, and HPRT [20]. Once normalized, data were statistically analyzed by GraphPad PRISM 4 Software (La Jolla, CA) using Mann-Whitney.

Tissue Microarray and Immunostaining Procedure

One cylinder of 1 mm was obtained from each tumor to build the tissue microarray (TMA; Beecher Instruments, Sun Prairie, WI) as previously described [21]. Sequential sections (4 mm thick) of the TMA were used to immunohistochemical detection of proteins ARL6IP5 (Gene Way Biotech, Inc, San Diego, CA; working dilution 1:400), AFAP1l2 (Protein Tech Group, Inc, Chicago, IL; working dilution 1:100), MCM2 (Protein Tech Group, Inc; working dilution 1:100) and SNRPD3 (Protein Tech Group, Inc; working dilution 1:100). The second-generation biotin-free polymeric system Advance (DAKO, Carpinteria, CA) was used for staining. All immunohistochemistry reactions were performed simultaneously to avoid any bias in the results due to the order of testing or differences in environmental conditions.

Immunostaining Analysis

All slides submitted to immunohistochemistry were labeled for blinded automated examination. All slides were digitalized using the Aperio System (Vista, CA), and the images provided by the software were exhibited on an LCD monitor under standardized contrast, focus, saturation, and white balance. Automated image quantification was performed using the images obtained. To evaluate the staining intensity, the Aperio image analysis system was used. This software identifies the immunohistochemical staining to be quantified by minimizing background-staining artifacts through image filters. Because the software recognizes positive nuclei or cytoplasm staining of all different intensities, the quantification was processed in each TMA spot automatically by the software. Numerical data of staining intensity average corresponding to each spot were exported to a Microsoft Excel (Seattle, WA) file for further statistical analysis. The software Prism 5 for Windows, version 5.02 (La Jolla, CA), was used for the immunohistochemistry analysis. The D'Agostino normality test was used to verify the data distribution pattern of each group. The *t* test was used when comparing two groups of normal distribution and the Mann-Whitney test was used when comparing two groups of nonparametric distribution to evaluate the differences between them.

Statistical Analysis

For data analysis, we used R (http://cran.r.project.org/), an open-source interpreted computer language for statistics, computation and graphics, and packages from the Bioconductor project (http://www.bioconductor.org), such as maigesPack. After image acquisition and quantification, spots with signal lower or equal to background were excluded from the analysis. Background-subtracted spot intensities were normalized by loess, using span equal to 0.4 and degree equal to 2. For identification of differentially expressed genes, the nonparametric test Mann-Whitney-Wilcoxon was applied, and *P* values were corrected by Bonferroni [22] and false discovery rate [23]. Using pairwise comparisons, we searched for differentially expressed genes considering three samples groups: BMTs, DTFs, and sarcomas (MMT). First, we compared BMTs *versus* DTFs (comparison A), followed by sarcomas *versus* DTFs (comparison B), and, finally, BMTs *versus* sarcomas samples (comparison C). For the last comparison, we ranked the genes according to their significance and observed their behavior on the first two comparisons.

Results

Aiming to identify genes potentially associated with local aggressiveness and metastatic potential of mesenchymal tumors, we determined the expression profile of a total of 102 fresh tumor samples. On the basis of their biological behavior and histopathology, tumors were grouped into three major categories: BMTs, tumors with local aggressiveness but with no metastatic potential, and sarcomas that have both local aggressiveness and metastatic potential (Figure 1). Next, we searched for differentially expressed genes in each of the three pairwise comparisons. A complete list with fold change and corrected *P* values is presented as Table W3.

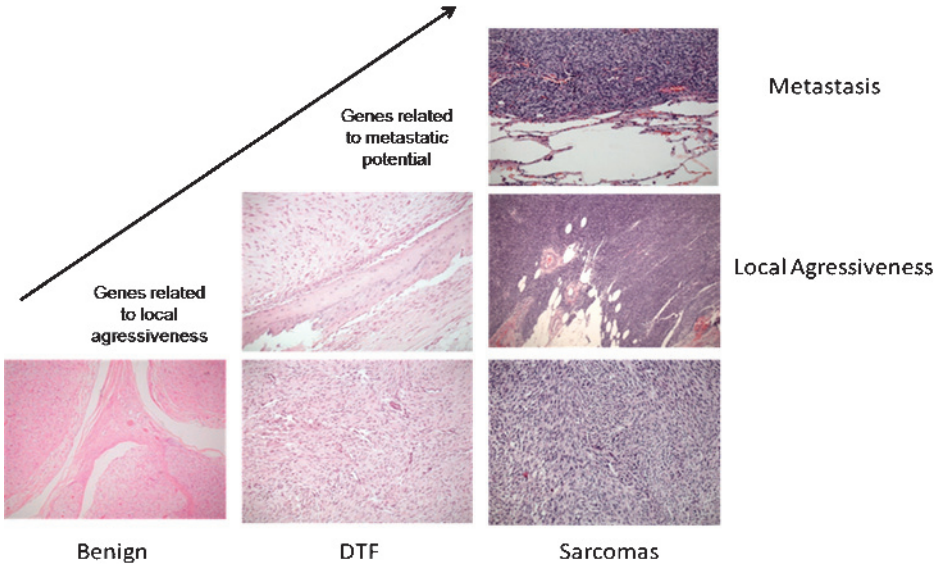

Figure 1. Histopathologic representation of diverse biological behavior in mesenchymal tumors (H&E staining).

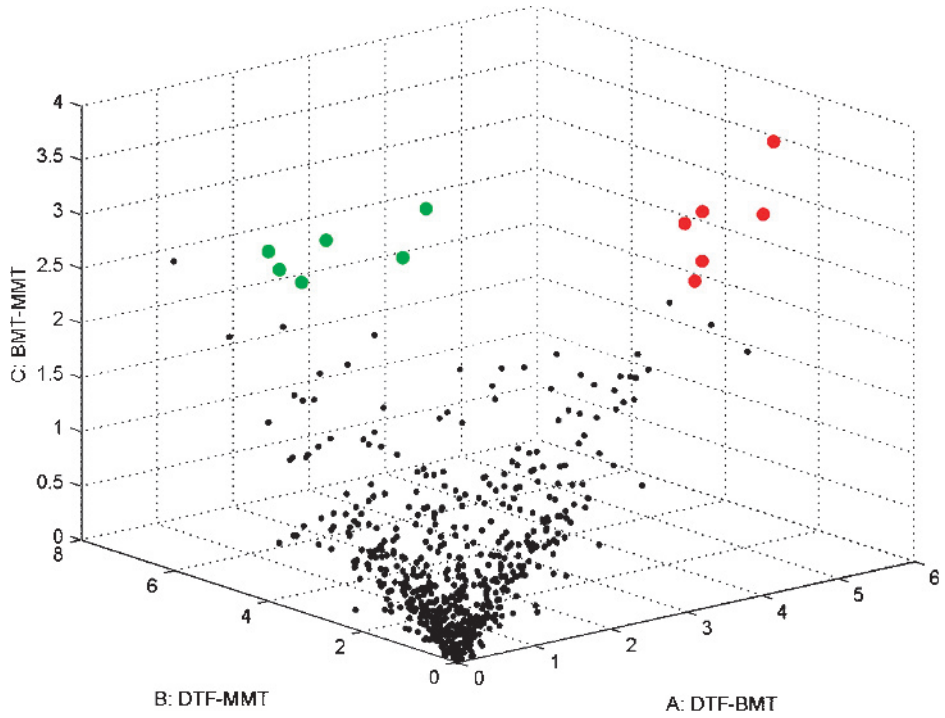

Figure 2. Three-dimensional scatter plot representing the expression of genes associated to local aggressiveness and metastasis. For each gene, we determined their nominal *P* values (Wilcoxon test, $-\log_{10}$) and plotted in a three-dimensional scatter plot. In red are the genes associated to local aggressiveness and in green are the genes associated to metastasis.

The genes involved in local aggressiveness could be interpreted as genes that are differently expressed between BMTs and DTF and also between BMTs and sarcomas, that is, differentially expressed in both comparisons C and A. The search for genes related to metastatic potential, using the same approach, could be interpreted as genes differentially expressed in comparison B, between sarcomas and DTF, as well as in comparison C, between BMTs and sarcomas.

For comparison C, we ranked the genes according to their statistical significance and observed their behavior on comparisons A and B. We noticed that the first 12 genes of this ranking showed a dichotomy when comparison A and B were made. Genes that were upregulated in A were downregulated in B and *vice versa*. Moreover, when plotted in a three-dimensional space according to their fold change, these could precisely discriminate between samples from A or B groups (Figure 2). Therefore, we focused further analysis on these genes. It is noteworthy that selection of these twelve genes was not based on differential expression but rather, on this opposite behavior in A and B comparisons. Hence, it is appropriate to consider their nominal *P* value. The genes related to local aggressiveness are *SNRPD3*, *MEGF9*, *SPTAN-1*, *AFAP1L2*, *ENDOD1*, and *SERPIN5*. The genes related to metastatic potential are *ZWINTAS*, *TOP2A*, *UBE2C*, *ABCF1*, *MCM2*, and *ARL6IP5*.

Differential Expression as a Function of Tumor Types: BMT, DTF, and MMT

The expression levels of these 12 genes in each tumor type (BMT, DTF, and MMT) are presented in Figure 3*A*, for genes potentially involved in local aggressiveness, and in Figure 3*B*, for genes potentially involved in metastasis. We also represented, by box plot, the expression profile of these 12 genes as a function of tumor types BMT, DTF and for MMT we segregate samples representing sarcomas of low, intermediate, or high grade (Figure W1). The same distribution was observed in Figure 3, and for some genes, such as *ARLP6IP5* and *TOP2A*, there is a gradual change from benign tumors to sarcomas of high grade. For others, such as *SERPIN5* and *SNRPD3*, there is a single-step change, suggesting that, if functionally involved, they would be associated with aggressiveness, either locally or at a distance.

Validation of Differential Expression

Validation of observed changes was done by quantitative PCR (Q-PCR) using RNA from samples used for the expression profile as well as from independent samples. For genes potentially related to local aggressiveness, we confirmed the differential expression for *AFAP1L2*, *MEGF9*, *ENDOD1*, and *SERPIN5* (Figure 4*A*).

For genes potentially related to metastasis, we confirmed the differential expression of *UBE2C*, *ZWINTAS*, *MCM2*, and *TOP2A* as being higher expressed in samples representing MMT compared with non-metastatic BMT and DTF samples (Figure 4*B*).

Validation of Protein Levels

We were able to determine protein levels for 4 of the 12 identified genes (*MCM2*, *ARLP6*, *AFAP1*, and *SNRPD3*). We have compared their protein expression levels by separating samples into the following groups: BMTs (leiomyomas, neurofibromas, and schwannomas), DTFs, and MMTs (synovial sarcomas, leiomyosarcomas, pleomorphic sarcomas, fibrosarcomas, liposarcomas, malignant peripheral nerve sheet tumor, GIST, and alveolar soft part sarcomas). For *MCM2* and *ARLP6* analysis (genes related to metastatic potential), we compared BMT plus DTF *versus* MMT, and for *AFAP1* and *SNRPD3* (genes related to local aggressiveness), we compared BMT *versus* DTF plus MMT.

We found a higher expression of *MCM2* in the BMT and DTF group in comparison with the MMT group ($P < .001$). For *ARLP6*, there was also a significantly higher protein expression in the BMT and DTF

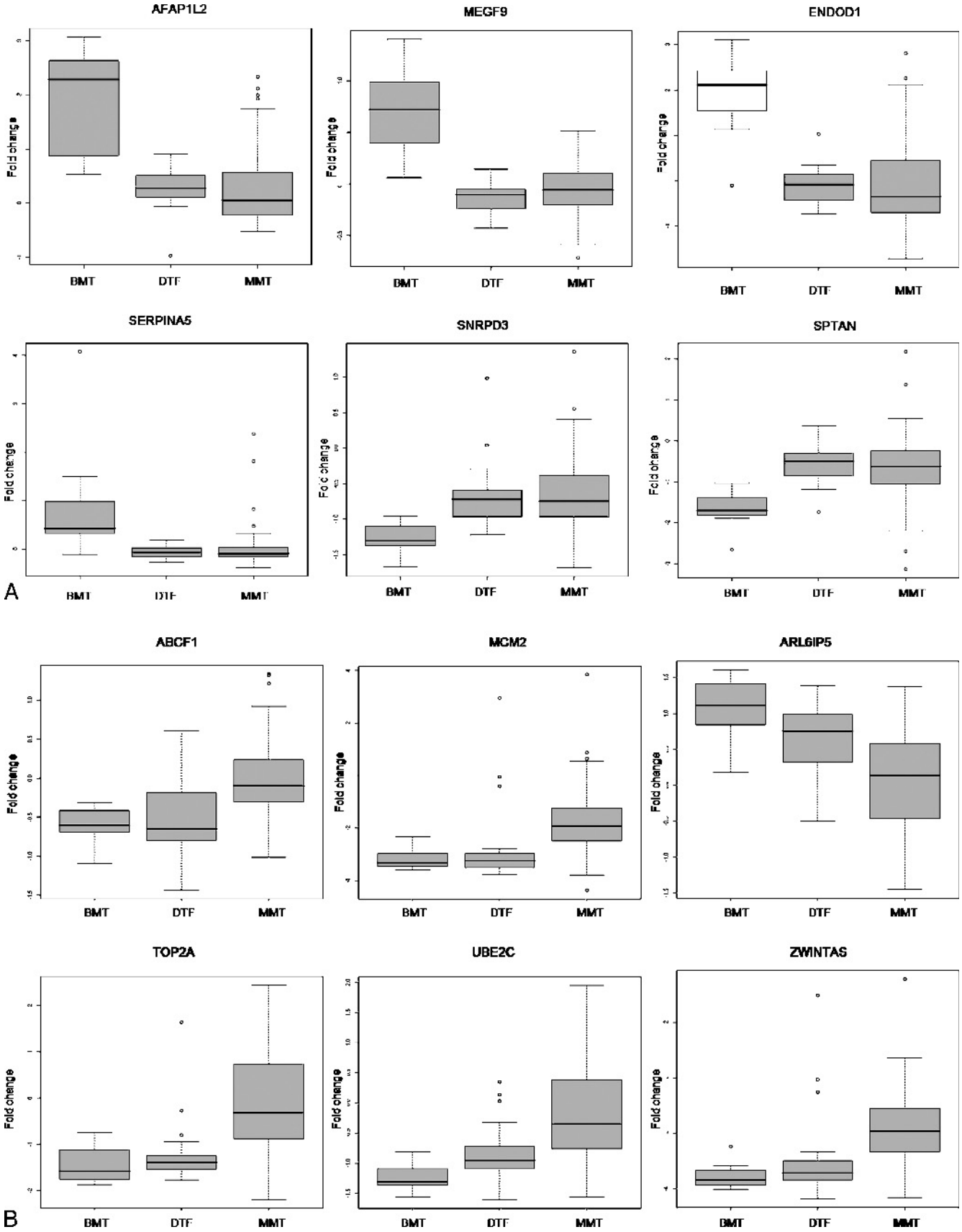

Figure 3. Representative box plot showing the expression (fold change) of the six genes related to local aggressiveness (*AFAP1L2*, *MEGF9*, *ENDOD1*, *SERPIN5*, *SNRPD3*, and *SPTAN*; A) and the six genes related to metastatic potential (*ABCF1*, *MCM2*, *ARL6IP5*, *TOP2A*, *UBE2C*, and *ZWINTAS*; B) in BMTs, DTFs, and MMTs. For all genes, the value for each pairwise comparison is described in Table W3.

group when compared to the MMT group ($P < .0001$). Conversely, there was a higher protein expression of *SNRPD3* in DTF and MMT than in BMT ($P = .013$). There was no significant difference expression of *AFAP1* between the two groups ($P = .343$; Figure 5).

Discussion

Soft tissue tumors encompass a myriad of different tumors with diverse cellular differentiation as well as different biological behavior. Although arising from distinct genetic alterations and/or signaling pathways, they can be grouped on the basis of their biological behavior, considering their local aggressiveness and metastatic potential. The molecular events related to these differences are yet unknown. The understanding of these mechanisms is important in discriminating those patients who will need systemic treatment from those that can be treated only locally. More than that, it also could guide for the development new drugs. In this study, we compared the expression profile of 102 tumor

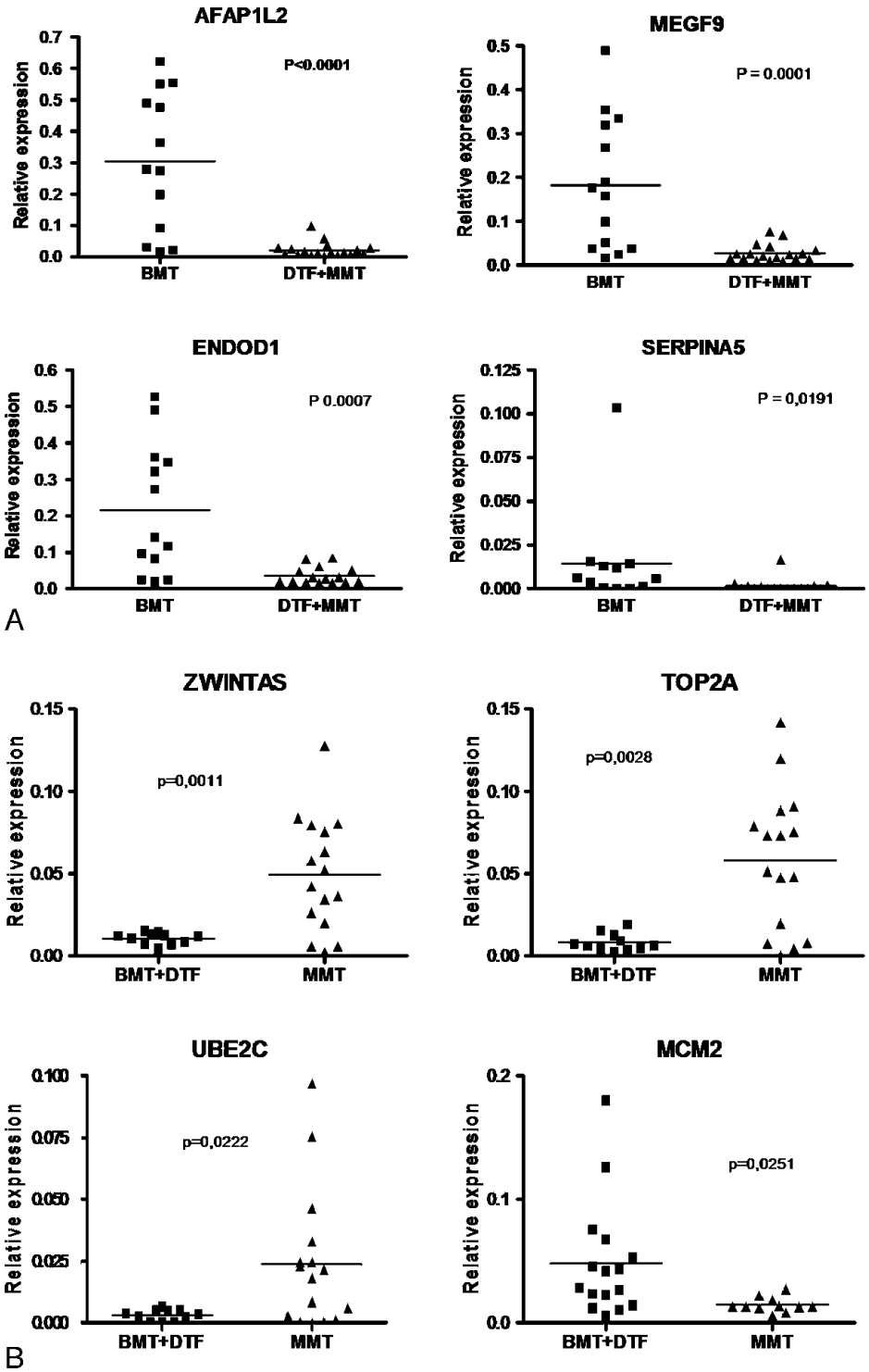

Figure 4. Validation by Q-PCR of the genes related to local aggressiveness (A) and metastasis (B). Total RNA used for Q-PCR was performed using SYBR green. *P* values were calculated by Mann-Whitney.

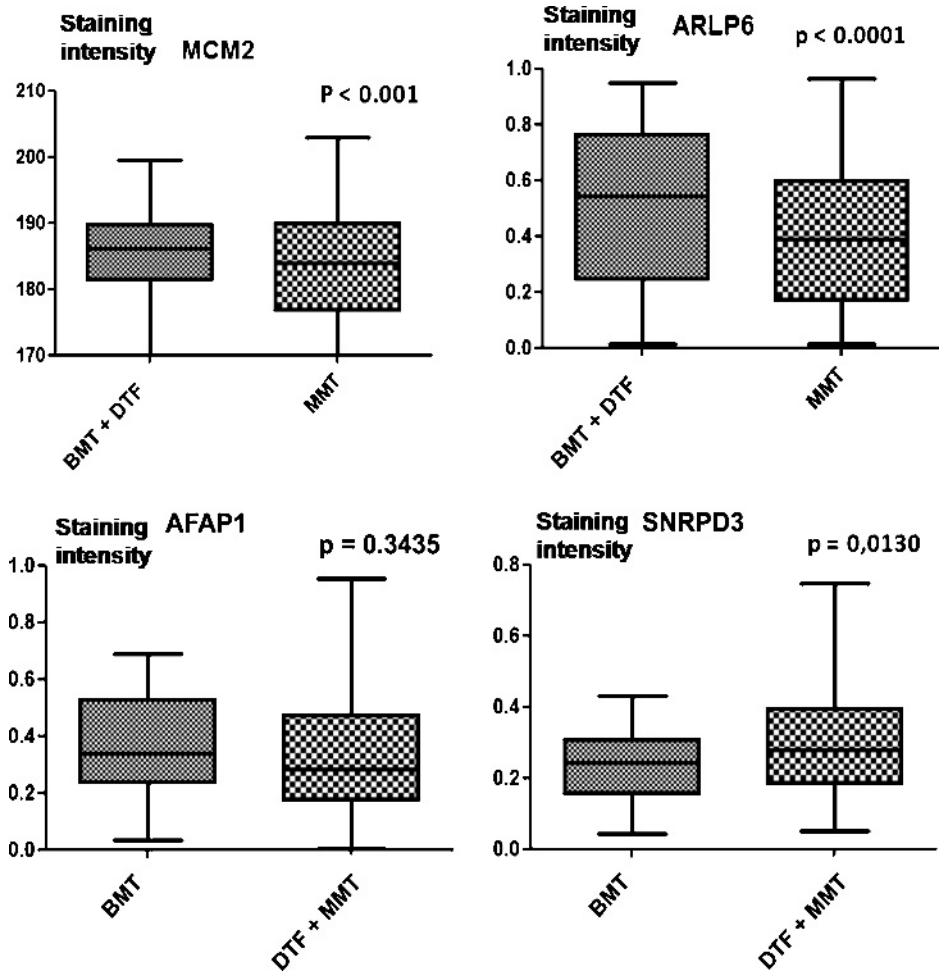

Figure 5. Representative box plot showing the protein expression of the two proteins related to local aggressiveness (AFAP1L2 and SNRPD3) and two proteins related to metastatic potential (MCM2 and ARL6IP5) in BMTs, DTFs, and MMTs.

samples representing soft tissue tumors. We identified a group of six genes that could be either markers or functional related to local aggressiveness (*SNRPD3*, *MEGF9*, *SPTAN-1*, *AFAP1L2*, *ENDOD1*, and *SERPIN5*) and another group of six genes that could be related to metastatic potential (*ZWINTAS*, *TOP2A*, *UBE2C*, *ABCF1*, *MCM2*, and *ARL6IP5*).

As recently reviewed by Chiang and Massagué [24], it is likely that cancer cells accumulate malignant function to promote expansion of the primary tumor, and this cumulative strategy might be necessary but not sufficient for the development of metastasis. Usually, initiation of metastasis requires alterations in genes related to local invasion (motility and extracellular matrix remodeling), angiogenesis, and epithelial-to-mesenchymal transition. For the establishment of metastasis, genes functionally related to vascular remodeling, immune evasion followed by alterations in organ-specific function might need to be altered. The set of genes that we identified in this article seems to follow this rationale. *SERPIN5*, *SPTAN-1*, and *MEGF9* are involved in cell invasion, tissue destruction, and cell motility, respectively, whereas *UBE2C*, *ARL6IP5*, *MCM2*, *TOP2A*, and *ABCF1* are involved in increase of metabolism, cell migration, cell cycle, cell proliferation, and malignant transformation, respectively. *AFAP1L2*, *SNRPD3*, *ZWINTAS*, and *ENDOD1* have never been related to cancer before but might play a role in tumor biology.

Since 2002, many groups reported the identification of predictors of tumor behavior such as responsiveness, local recurrence, or metastatic potential [24–30]. For example, van 't Veer et al. [25] defined a set of 71 genes that, according to their expression profile, could define the need for adjuvant in patients with early-stage breast carcinoma. Also, Ramaswamy et al. [26] compared gene expression profile of primary adenocarcinomas and unmatched metastasis and revealed that primary and metastatic tumors share a set of genes commonly altered suggesting that aggressiveness is an early event during tumor development.

In sarcomas, the pattern of metastasis differs from carcinomas. Sarcomas rarely metastasize to lymph nodes, and the most frequent sites of metastasis are lungs follow by liver (hematogenic metastasis). Because soft tissue sarcomas are of mesodermal origin, probably the molecular events related to metastasis of these tumors may be distinct from tumors of other embryonic origins.

There are very few reports correlating gene expression profile and metastasis in sarcomas. Lee et al. [27] have identified a gene expression signature associated with metastasis in leiomyosarcoma that allowed prediction of the future development of metastases. The most discriminating genes are those encoding for proteins involved in tumor development and invasion, especially cell growth and transition through cell cycle. Ren et al. [28] identified a 92-gene signature in

11 leiomyosarcomas that separated high-grade metastatic tumors from low-grade ones. More recently, Francis et al. [29] suggest a prognostic profile modulated at least in part by hypoxia in a large series of highly malignant soft tissue tumors of mixed types. Using cDNA microarray, Nakano et al. [30] found seven genes that were differently expressed between high- and low-grade metastatic sublines of human osteosarcoma cell lines. Among those genes, five of them (*AXL*, *TGFA*, *COLL7A1*, *WNT5A*, and *MKK6*) were associated with adherence, motility, and/or invasiveness, suggesting that differences in motility/invasiveness and adherence are highly involved in the metastatic process in osteosarcoma. In this same study, proliferative activity showed no obvious correlation with the metastatic potential.

Among the genes possibly involved in local aggressiveness, *SERPIN5* was identified as inhibited in locally aggressive as well as in metastatic tumors compared with benign tumors. Also known as *PCI* (protein C inhibitor), *SERPIN5* is known to regulate the activity of the serine proteases involved in blood coagulation, wound healing, and tumor metastasis [31,32]. For instance, Wakita et al. [33] demonstrated that PCI antigen level is significantly lower in renal cell carcinoma (RCC) tissue than in nontumor kidney tissue, and accordingly, the expression of PCI messenger RNA was detected in normal renal proximal tubular epithelial cells but not in RCC or in an RCC cell line (Caki-1 cells). Also, *in vitro* invasiveness of Caki-1 cells transfected with a PCI expression vector was significantly decreased compared with mock-transfected Caki-1 cells by the addition of anti-PCI.

Protein C inhibitor also inhibits breast cancer cell growth, metastasis, and angiogenesis independently of its protease inhibitory activity [34]. Urokinase-type plasminogen activator and plasminogen activator inhibtor-1 (PAT-1) are known to be associated with a poor prognosis in breast cancer. PAI-3 is expressed in human breast tumors, and elevated levels of PAI-3 could be a positive prognostic factor in this disease. A potential mechanism for the contribution of *PAI-3* to a positive long-term outcome may involve suppression of tumor invasion through protease inhibition in stroma [31]. Hence, our observation that *SERPIN5* expression is diminished as a function of tumor aggressiveness corroborates all these findings and, as suggested by others, its modulation might be beneficial for local disease control.

MEGF9 (multiple epidermal growth factor [EGF]–like domains) is a novel transmembrane protein with multiple EGF-like repeats predominantly expressed in the developing and adults central nervous system and peripheral nervous system. EGF-like domains are major modular components present in many proteins of the extracellular matrix [35]. Mutation in these proteins has been related to several disorders such as Marfan syndrome [36] and leukoencephalopathy [37]. We found a higher expression of *EGFL* in BMT. This is, to the best of our knowledge, the first observation suggesting that decreased expression of *MEGF9* in mesenchymal tumors may be associated to tumor local invasion.

There are very few data about the role of the other three genes differentially expressed in local aggressiveness (*SNRPD3*, *AFAP1L2*, and *SPTANII/SPETRIN*) and cancer. *SNRPD3*, also known as *SMD3*, plays an essential role in the formation of small nuclear ribonucleoprotein particles by binding to small nuclear RNA and participating in a network of protein interactions [38] and has never been related to cancer before. *AFAP1L2* is an adaptor protein for signal transduction. Downregulation of *AFAP1L2* causes a reduction of c-Src activity, IL-8 production, EGF-induced phosphorylation of AKT and GSK3β in human lung epithelial cells, altering the cell cycle [39]. Finally, nonerythroid α spectrin *SPTAN-1* (ALPHA II Spectrin) is shown to be involved in DNA repair. It is related to tumorigenesis in ovarian cancer [40], and *SPTAN-1* gene was significantly higher in gastric cancer tissue than in normal gastric mucosa tissue and dysplasia tissue [41].

As for metastatic potential, we identified *TOP2A* as a gene whose overexpression was observed in tumors with metastatic potential. This observation is in agreement with several recent reports including one by Kozari et al. [42] describing *TOP2A* as one of the most valuable markers for aggressive prostate cancer. We also observed overexpression of *UBE2C*, a gene frequently found upregulated in tumors with malignant potential and metastasis. Okamoto et al. [43] found a higher expression of *UBE2C* in diverse cancer cell lines and primary tumors, mainly carcinomas, compared with corresponding normal tissues. Takahashi et al. [44] observed its overexpression in advanced colon cancer with liver metastasis. In sarcomas, Arvand et al. [45] demonstrated that *UBE2C* is upregulated in NIH3T3 cells transformed with *EWS-FLI1* fusion gene.

MCM2 is a component of the DNA replication licensing complex, which marks DNA replication origins during G– of cell cycle for use in subsequent S-phase [46]. An increased expression of *MCM2* had also been reported in several human tumors when compared to the corresponding nontumor tissue such as hepatocellular carcinoma and

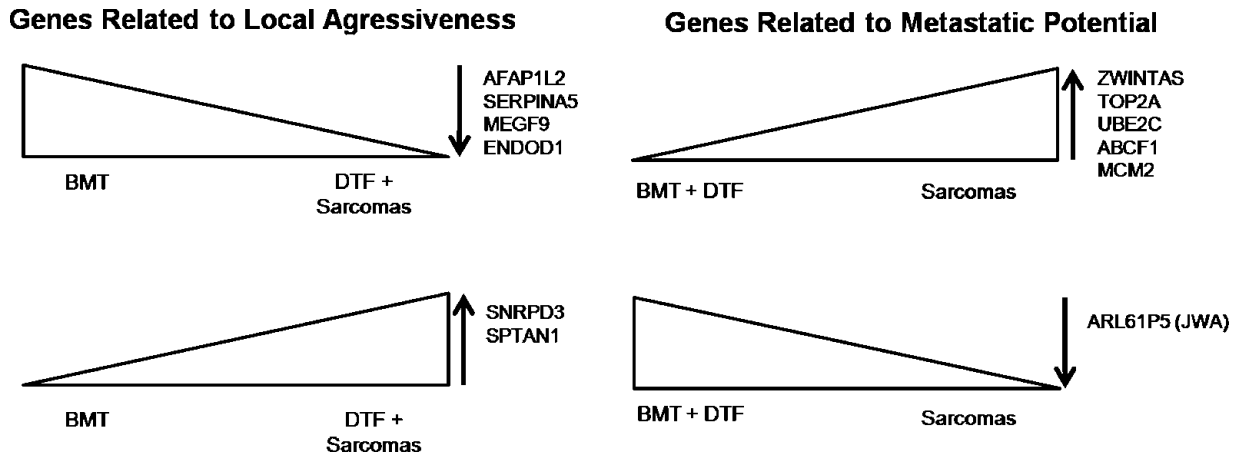

Figure 6. Schematic representation of selected genes based on a dichotomy in their expression profile related to local aggressiveness and metastatic potential in BMTs, DTFs, and MMTs.

pancreatic adenocarcinoma [47,48]. Sington et al. [49] showed that *MCM2* is more expressed in myxofibrosarcoma, having a higher histologic grade. Huang et al. [50] also found a relation between increased expression of *MCM2* and adverse prognostic in myxofibrosarcoma. We also found a higher expression in sarcomas of all types than benign soft tissue tumors and DTF.

One interesting finding was the overexpression of *ARL6IP5* (JWA) in tumors without metastatic potential compared with aggressive sarcomas. *ARL6IP5* (JWA) codes a microtubule-associated protein that is essential for the rearrangement of F-actin cytoskeleton and activation of mitogen-activated protein kinase cascades induced by arsenic trioxide [51] Therefore, it plays a role in invasion and metastasis. In HeLa, B16, and HCCLM3 cancer cells, overexpression of JWA, inhibited cellular migration, and induced deficiency of JWA in HeLa cells implicate an increase in cell migration [52]. Other studies showed that polymorphisms on JWA gene exon 2 was related to leukemia, gastric, esophageal, and bladder carcinomas [53–55]. Our data suggest that the down-regulation of JWA by sarcomas may be one of the factors responsible for the more aggressive behavior and metastatic potential in soft tissue tumors.

Another gene related with cell migration is *ZWINTAS*. It is a key member of the apicobasal Crumbs polarity complex. Cell polarity is induced and maintained by the separation of the apical and basolateral domains through specialized cell-cell junctions. The Crumbs protein and its binding partners are involved in the formation and stabilization of adherens junctions [56,57]. Cell polarity is a key process in cell migration, and *ZWINTAS* has never been related to tumorigenesis and tumor behavior before.

Finally, *ABCF1* was highly expressed in sarcomas when compared to BMTs and fibromatosis. This gene is one of the 49 members of human ATP-binding cassettes transporters. High expression levels of these transporters have already been reported in several malignant tumors, including sarcomas, and are related to multidrug resistance [58].

Hence, based on the expression of genes previously identified as altered, and functionally related to local aggressiveness and metastatic behavior, our data point to a set of genes with known function but not previously associated with the biology of mesenchymal tumors. Interestingly, the genes described herein were selected based on a dichotomy in their expression profile and, as summarized in Figure 6, seem to acquire altered correlation as the disease progresses as suggested by Chiang and Massagué [24]. Functionally, an orchestrated balance among them might be critical for maintaining cellular and tissue homeostasis and, as such, might represent new markers or targets for the management of sarcomas.

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Table W1. Description of the 102 Samples Used in the c-DNA Microarray and Q-PCR Experiments.

Sample	Diagnosis	Histologic Grade	Category	Localization	cDNA Microarray	Q-PCR
FTS201	Fibroma of tendon sheath	NA	BMT	Finger	No	Yes
LM45	Leiomyoma	NA	BMT	Cervical	Yes	Yes
LM46	Leiomyoma	NA	BMT	Pelvic	Yes	Yes
NF170	Neurofibroma	NA	BMT	Thigh	Yes	Yes
NF96	Neurofibroma	NA	BMT	Cervical	Yes	No
NF207	Neurofibroma	NA	BMT	Head	No	Yes
NF208	Neurofibroma	NA	BMT	Neck	No	Yes
NF209	Neurofibroma	NA	BMT	Parotid gland	No	Yes
NF210	Neurofibroma	NA	BMT	Neck	No	Yes
NF211	Neurofibroma	NA	BMT	Neck	No	Yes
NF213	Neurofibroma	NA	BMT	Neck	No	Yes
NF214	Neurofibroma	NA	BMT	Shoulder	No	Yes
NF215	Neurofibroma	NA	BMT	Orbit	No	Yes
NF98	Neurofibroma	NA	BMT	Thigh	Yes	No
SH155	Schwannoma	NA	BMT	Leg	Yes	No
SH158	Schwannoma	NA	BMT	Retroperitoneum	Yes	Yes
SH216	Schwannoma	NA	BMT	Leg	No	Yes
FM165	Desmoid-type fibromatosis	NA	DTF	Thigh	Yes	No
FM166	Desmoid-type fibromatosis	NA	DTF	Neck	Yes	No
FM168	Desmoid-type fibromatosis	NA	DTF	Abdominal	Yes	No
FM193	Desmoid-type fibromatosis	NA	DTF	Thigh	Yes	No
FM195	Desmoid-type fibromatosis	NA	DTF	Thoracic	Yes	No
FM196	Desmoid-type fibromatosis	NA	DTF	Abdominal	Yes	No
FM197	Desmoid-type fibromatosis	NA	DTF	Arm	Yes	No
FM25	Desmoid-type fibromatosis	NA	DTF	Mandible	Yes	Yes
FM26	Desmoid-type fibromatosis	NA	DTF	Mandible	Yes	Yes
FM27	Desmoid-type fibromatosis	NA	DTF	Abdominal	Yes	No
FM28	Desmoid-type fibromatosis	NA	DTF	Leg	Yes	Yes
FM29	Desmoid-type fibromatosis	NA	DTF	Thigh	Yes	No
FM30	Desmoid-type fibromatosis	NA	DTF	Abdominal	Yes	No
FM31	Desmoid-type fibromatosis	NA	DTF	Thoracic	Yes	No
FM32	Desmoid-type fibromatosis	NA	DTF	Pelvis	Yes	No
FM33	Desmoid-type fibromatosis	NA	DTF	Buttock	Yes	Yes
FM34	Desmoid-type fibromatosis	NA	DTF	Thoracic	Yes	Yes
FM38	Desmoid-type fibromatosis	NA	DTF	Buttock	Yes	Yes
FM97	Desmoid-type fibromatosis	NA	DTF	Head and neck	Yes	Yes
FM202	Desmoid-type fibromatosis	NA	DTF	Abdominal	No	Yes
FM203	Desmoid-type fibromatosis	NA	DTF	Knee	No	Yes
FM204	Desmoid-type fibromatosis	NA	DTF	Abdominal	No	Yes
FM205	Desmoid-type fibromatosis	NA	DTF	Scapular	No	Yes
FM206	Desmoid-type fibromatosis	NA	DTF	Abdominal	No	Yes
SA113	Alveolar soft part sarcoma	High	MMT	Head and neck	Yes	No
SA178	Alveolar soft part sarcoma	High	MMT	Forearm	Yes	Yes
FS164	Fibrosarcoma	High	MMT	Thigh	Yes	Yes
FS35	Fibrosarcoma	Intermediate	MMT	Scalp	Yes	Yes
FS37	Fibrosarcoma	High	MMT	Scapular	Yes	No
FS39	Fibrosarcoma	Low	MMT	Scapular	Yes	No
FS40	Fibrosarcoma	High	MMT	Leg	Yes	Yes
FS41	Fibrosarcoma	High	MMT	Dorsum	Yes	Yes
GI159	GIST	Low	MMT	Rectum	Yes	Yes
GI169	GIST	Low	MMT	Stomach	Yes	Yes
GI43	GIST	Low	MMT	Duodenum	Yes	No
LES136	Leiomyosarcoma	High	MMT	Orbit	Yes	No
LES172	Leiomyosarcoma	High	MMT	Prostate	Yes	No
LES173	Leiomyosarcoma	High	MMT	Uterus	Yes	Yes
LES182	Leiomyosarcoma	High	MMT	Thigh	Yes	No
LES48	Leiomyosarcoma	Low	MMT	Pelvis	Yes	Yes
LES49	Leiomyosarcoma	Low	MMT	Retroperitoneum	Yes	No
LES51	Leiomyosarcoma	Low	MMT	Thigh	Yes	No
LES52	Leiomyosarcoma	Low	MMT	Perineum	Yes	No
LES53	Leiomyosarcoma	Intermediate	MMT	Inguinal region	Yes	Yes
LES56	Leiomyosarcoma	Intermediate	MMT	Pelvis	Yes	No
LES57	Leiomyosarcoma	Low	MMT	Mandible	Yes	No
LES58	Leiomyosarcoma	High	MMT	Vagina	Yes	Yes
LES59	Leiomyosarcoma	High	MMT	Uterus	Yes	No
LES60	Leiomyosarcoma	High	MMT	Head and neck	Yes	Yes
LES61	Leiomyosarcoma	High	MMT	Uterus	Yes	No
LES62	Leiomyosarcoma	High	MMT	NA	Yes	No
LES63	Leiomyosarcoma	High	MMT	Uterus	Yes	No
LES64	Leiomyosarcoma	High	MMT	Retroperitoneum	Yes	No
LES65	Leiomyosarcoma	High	MMT	Thigh	Yes	No
LPS75	Lipoblastic liposarcoma	Low	MMT	Retroperitoneum	Yes	No
TM160	MPNST	High	MMT	Dorsum	Yes	No
TM179	MPNST	Low	MMT	Head and neck	Yes	No

Table W1. (*continued*)

Sample	Diagnosis	Histologic Grade	Category	Localization	cDNA Microarray	Q-PCR
TM194	MPNST	Low	MMT	Head and neck	Yes	No
TM198	MPNST	High	MMT	Head and neck	Yes	No
TM200	MPNST	Low	MMT	Leg	Yes	No
LPS81	Myxoid liposarcoma	Low	MMT	Leg	Yes	No
LPS89	Myxoid liposarcoma	Low	MMT	Thigh	Yes	No
SP122	Pleomorphic sarcoma	High	MMT	Forearm	Yes	Yes
SP124	Pleomorphic sarcoma	High	MMT	Mandible	Yes	No
SP125	Pleomorphic sarcoma	High	MMT	Retroperitoneum	Yes	Yes
SP126	Pleomorphic sarcoma	High	MMT	Thigh	Yes	Yes
SP127	Pleomorphic sarcoma	High	MMT	Skin/breast	Yes	Yes
SP131	Pleomorphic sarcoma	High	MMT	Neck	Yes	Yes
SP132	Pleomorphic sarcoma	High	MMT	Leg	Yes	No
SP134	Pleomorphic sarcoma	High	MMT	Retroperitoneum	Yes	No
SP135	Pleomorphic sarcoma	High	MMT	Retroperitoneum	Yes	No
SP138	Pleomorphic sarcoma	High	MMT	Thigh	Yes	No
SP161	Pleomorphic sarcoma	High	MMT	Mandible	Yes	No
SP174	Pleomorphic sarcoma	High	MMT	Arm	Yes	No
SP175	Pleomorphic sarcoma	High	MMT	Thigh	Yes	No
SP186	Pleomorphic sarcoma	High	MMT	Arm	Yes	No
SP188A	Pleomorphic sarcoma	High	MMT	Thigh	Yes	No
SP190A	Pleomorphic sarcoma	High	MMT	Thigh	Yes	No
SP191	Pleomorphic sarcoma	High	MMT	Buttock	Yes	No
LPS68	Round cell liposarcoma	High	MMT	Thigh	Yes	No
LPS137	Round cell liposarcoma	High	MMT	Thigh	Yes	No
SS140	Synovial sarcoma	High	MMT	Thigh	Yes	Yes
SS142	Synovial sarcoma	High	MMT	Thigh	Yes	No
SS143	Synovial sarcoma	High	MMT	Thigh	Yes	Yes
SS144	Synovial sarcoma	High	MMT	Elbow	Yes	No
SS145	Synovial sarcoma	High	MMT	Arm	Yes	No
SS147	Synovial sarcoma	High	MMT	Leg	Yes	No
SS148	Synovial sarcoma	High	MMT	Arm	Yes	No
SS149	Synovial sarcoma	High	MMT	Arm	Yes	No
SS152	Synovial sarcoma	High	MMT	Inguinal	Yes	No
SS153	Synovial sarcoma	High	MMT	Arm	Yes	No
SS154	Synovial sarcoma	High	MMT	Head and neck	Yes	No
SS157	Synovial sarcoma	High	MMT	Elbow	Yes	No
SS162	Synovial sarcoma	High	MMT	Thigh	Yes	No
SS176	Synovial sarcoma	High	MMT	Thigh E	Yes	No
SS177	Synovial sarcoma	High	MMT	Paraspinal	Yes	Yes
SS183	Synovial sarcoma	High	MMT	Knee	Yes	No
SS192	Synovial sarcoma	High	MMT	Hand	Yes	No
SS199	Synovial sarcoma	High	MMT	Leg	Yes	No
SS36	Synovial sarcoma	High	MMT	Thigh	Yes	No

Table W2. Primer Sets Used in Q-PCR Experiment.

Gene Name	Primers Sequence	Amplicon Length (bp)
AFAP1	5′-GGTCGTGGTCACAGGCAAA-3′	63
	5′-GCTCCTTTCTTCTCCCATTCCT-3′	
ENDOD	5′-CTGGTGGAGCCGCAGATC-3′	64
	5′-GGCCTCTGCCTCATTAATCG-3′	
MEGF	5′-GTTCACGACCTCGAGGGAAA-3′	65
	5′-GTAGAACCTTCAGGTGTTGGAAGAA-3′	
SERPIN	5′-TCTGTCCGGCATCAGCAA-3′	62
	5′-CCACAGCTTTGTGCACCATCT-3′	
MCM2	5′-CACACAGAAGTTCAGCGTCATG-3′	60
	5′-AATGAAAGGTAGCGGGCAAA-3′	
TOP2A	5′-CCTAAAAAGAATGTGACAGTGAAGAAGA-3′	65
	5′-CCGGTAGTGGAGGTGGAAGA-3′	
UBE2E	5′-GCCGAGCTCTGGAAAAACC-3′	64
	5′-CTGGTGACCTGCTTTGAGTAGGT-3′	
ZWINTAS	5′-TGGAGGACAGCAGCATGGA-3′	61
	5′-TTGGGAGGTGAGGGAAGTCA-3′	
SNRPD3	5′-TGGAGGACAGCAGCATGGA-3′	75
	5′-TTGGGAGGTGAGGGAAGTCA-3′	
SPTAIN1	5′-GCTCAGAGGGAAAGCCTTACG-3′	63
	5′-CTTGTTCCCGGGTCAGGTT-3′	
ABCF1	5′-CACGCCCACACCATCCA-3′	57
	5′-CACAACTCGCGCCTTCTGA-3′	
ARL61P5	5′-TGGAGGAGTCATGGTCTTTGTG-3′	67
	5′-CGATGCATGGATAAACATCAACA-3′	

Table W3. Complete List with Fold Change and Corrected *P* Values of All Genes Are Available at http://www.maiges.org/sarcomaFibromatosis/.

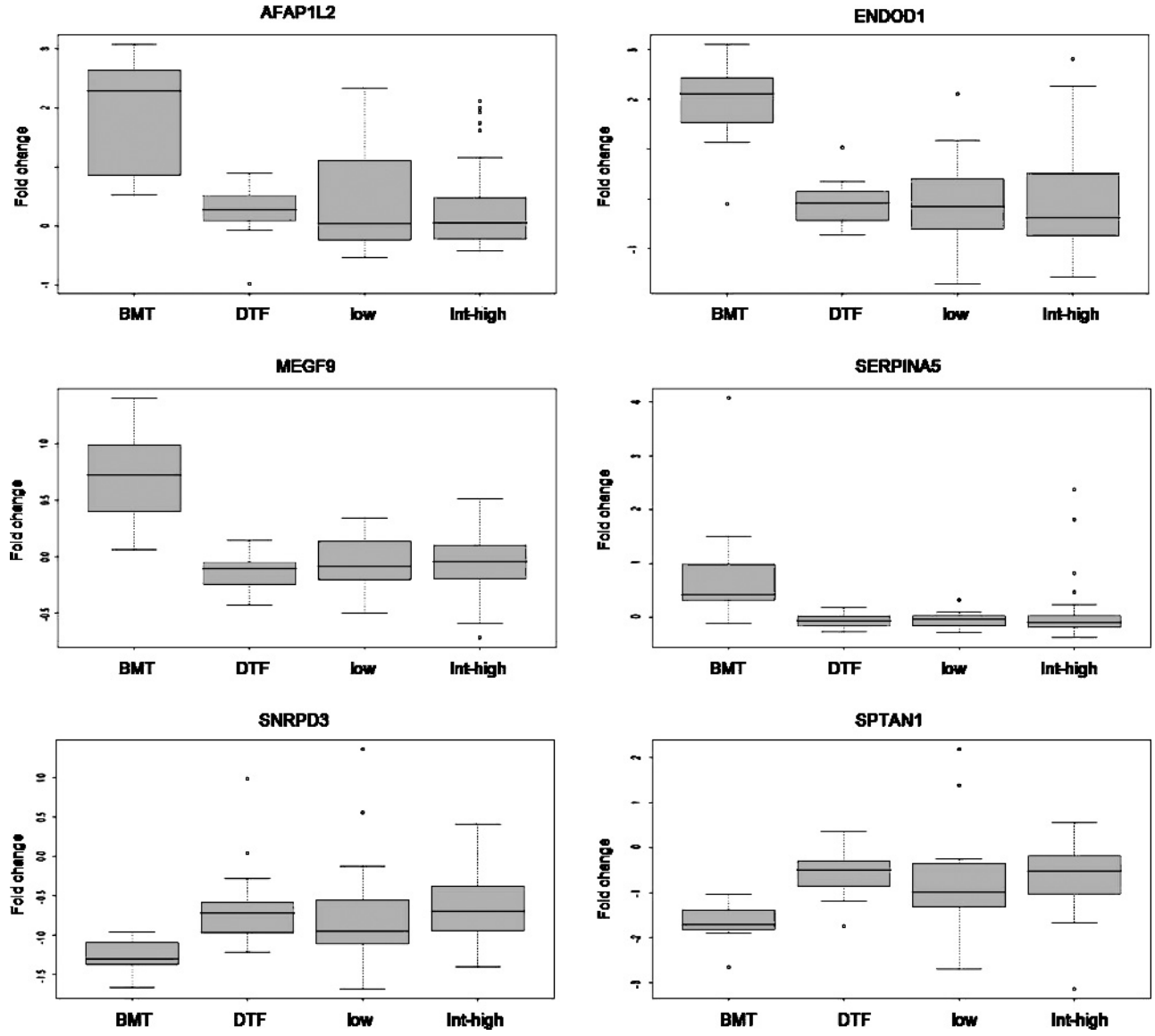

Figure W1. Representative box plot showing the expression (fold change) of the six genes related to local aggressiveness (*AFAP1L2*, *MEGF9*, *ENDOD1*, *SERPIN5*, *SNRPD3*, and *SPTAN*; and the six genes related to metastatic potential (*ABCF1*, *MCM2*, *ARL6IP5*, *TOP2A*, *UBE2C*, and *ZWINTAS*; in BMTs, DTFs, and low-, intermediate-, and high-grade sarcomas. For all genes, the value for each pairwise comparison is described in Table W3.

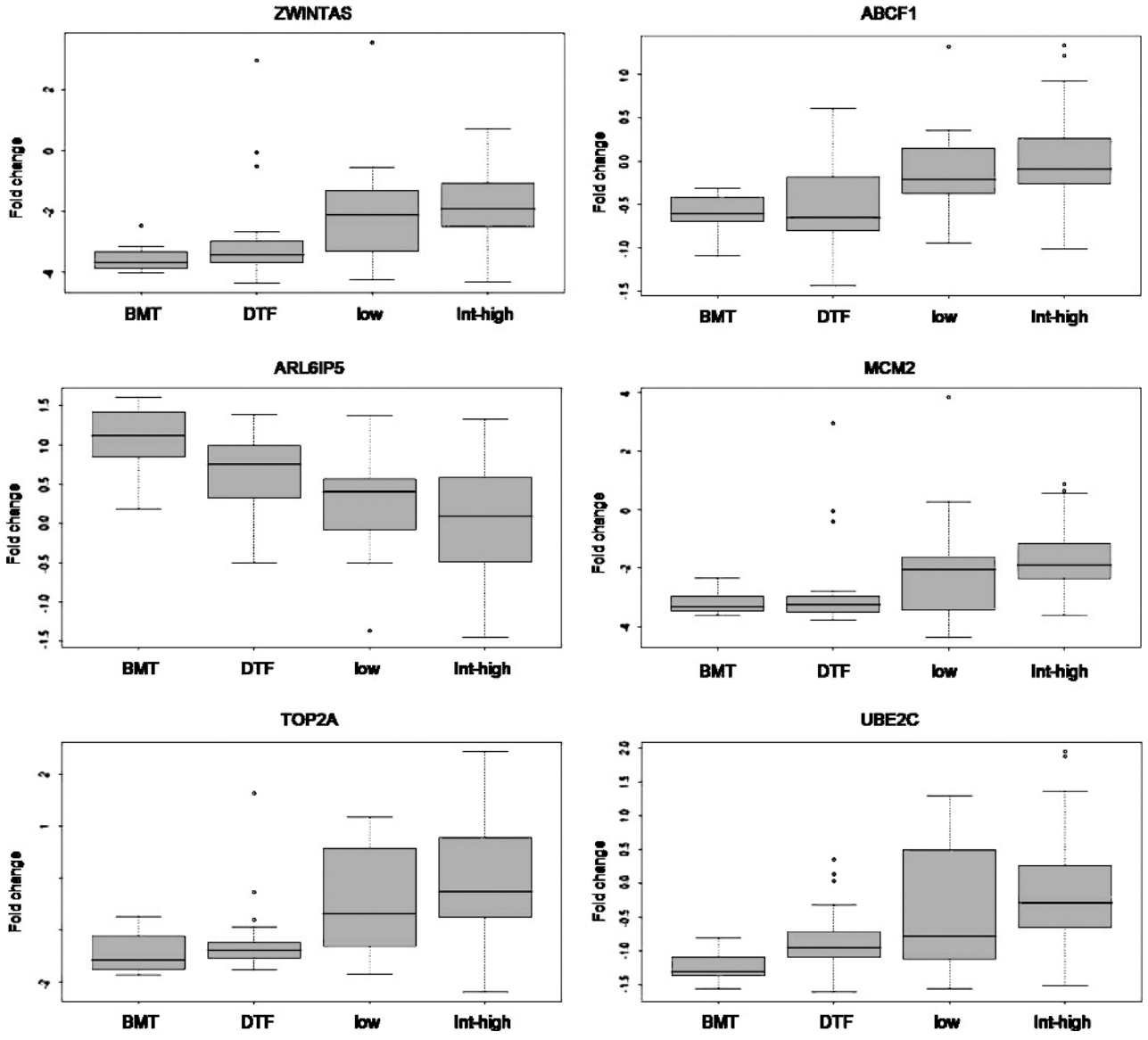

Figure W1. (*continued*).