ANÁLISE DE GENES DIFERENCIALMENTE EXPRESSOS EM TUMORES DE PARTES MOLES

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RESUMO

Cunha IW. **Análise de genes diferencialmente expressos em tumores de partes moles**. São Paulo; 2009. [Tese de Doutorado-Fundação Antonio Prudente]

Os tumores de partes moles são raros, de alta morbidade e mortalidade dependendo do subtipo histológico. Os eventos moleculares relacionados aos seus comportamentos como agressividade local e metástase permanecem desconhecidos. Neste estudo nós comparamos o perfil da expressão gênica de 102 amostras de tumores mesenquimais de diversos tipos histológicos e comportamentos biológicos através de cDNA microarray. Nós identificamos um grupo de seis genes relacionados funcionalmente à agressividade local (*SNRPD3, MEGF9, SPTAN-1, AFAP1L2, ENDOD1 e SERPIN5*), e outro grupo de seis genes relacionados à potencial metastático (*ZWINTAS, TOP2A, UBE2C, ABCF1, MCM2 e ARL6IP5*). Além disto, o reconhecimento de assinaturas moleculares trouxe melhor entendimento da patogênese destas neoplasias. Através de análise não supervisionada, o perfil de expressão gênica global dos casos analisados, foi capaz de diferenciar três grandes grupos de tumores de partes moles: fibromatoses (FM), sarcomas sinoviais (SS) e os demais tipos. Na procura de classificadores pelo discriminador linear de Fisher, encontramos 3 trios de genes capazes de separar os sarcomas sinoviais com 100% de acerto dos outros subtipos tumorais e 92 pares de genes capazes de separar as fibromatoses dos fibrossarcomas com 100% de acerto. Em outra abordagem avaliamos através das técnicas de cDNA microarray, Q-PCR e imunoistoquímica, a expressão de GFAP em diversos tipos histológicos de tumores mesenquimais. Os achados encontrados nos mostraram que a expressão de GFAP é decorrente da capacidade de diferenciação cartilaginosa presente em alguns sarcomas.

SUMMARY

Cunha IW. **[Expression profile of soft tissue tumors]**. São Paulo; 2009. [Tese de Doutorado-Fundação Antonio Prudente]

Soft tissue mesenchymal tumors represent a group of neoplasias with different histological and biological presentations varying from benign, locally confined to very aggressive and metastatic tumors. The molecular mechanisms responsible for such differences are still unknown. 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. SNRPD3, MEGF9, SPTAN-1, AFAP1L2, ENDOD1 and SERPIN5 were related to local agressiveness while ZWINTAS, TOP2A, UBE2C, ABCF1, MCM2 and ARL6IP5 were related to metastasis. Searching for molecular signature in mesenchymal tumors, we identified trios and pairs of genes capable to discriminate histological groups between all cases analyzed. We also evaluated the gene and protein expression of GFAP using Q-PCR and IHC in several cases of soft tissue and bone tumors and found that GFAP is a potential marker for tumors with cartilaginous differentiation.

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1 INTRODUÇÃO

1.1 ASPECTOS GERAIS

Os tumores de partes moles são raros correspondendo a menos que 10% de todas as neoplasias (FLETCHER et al. 2002) As neoplasias benignas são dez vezes mais freqüentes que as malignas e a grande maioria curável. As neoplasias de partes moles malignas (sarcomas) correspondem a menos de 1% de todos os cânceres, mas apesar da baixa incidência, possuem uma enorme importância devido a suas altas taxas de morbidade e mortalidade. Estima-se 10.390 casos novos de sarcoma para 2008 nos EUA pela American Cancer Society (ACS) com cerca de 3.680 óbitos. Estas neoplasias representam um grande desafio para médicos patologistas, oncologistas clínicos, cirurgiões e radioterapeutas, uma vez que diagnosticá-los corretamente nem sempre é uma tarefa fácil, geramente são mau respondedores a quimio e radioterapia, e exigem grande habilidade cirúrgica para sua completa ressecção.

Os sarcomas ocorrem em todas as idades. O que varia, é o tipo histológico mais frequente em cada faixa etária. Há sarcomas usualmente pediátricos e outros presentes apenas na vida adulta. Ocorrem por todo o organismo, com variações também decorrentes de subtipos histológicos.

A etiologia da grande maioria dos tumores de partes moles é desconhecida. Fatores como radiação, infecções virais, imunodeficiências

parecem ter implicações em alguns tipos de sarcomas, mas a grande maioria parece se desenvolver sem fatores ambientais aparentes.

Mais recentemente, com o advento de estudos moleculares, a melhor caracterização destas neoplasias vem se tornando possível.

1.1.1 Classificação histológica

Na tentativa de melhor caracterização destes tumores, várias classificações já foram propostas para melhor agrupamento destas neoplasias. Atualmente, eles são divididos de acordo com sua diferenciação celular em vários subgrupos (FLETCHER et al. 2002):

- Tumores Adipocíticos;
- Tumores Fibroblásticos/Miofibroblásticos;
- Tumores chamados de Fibrohistiocíticos;
- Tumores Musculares lisos;
- Tumores Pericíticos;
- Tumores Musculares esqueléticos;
- Tumores Vasculares;
- Tumores Condro-ósseos;
- Tumores de Diferenciação incerta.

Existem vários subtipos em cada um destes grupos, classificados de acordo com características morfológicas e de comportamento clínico.

Apesar da tentativa de discriminar os diversos tipos histológicos, clinicamente as opções de tratamentos são restritas, e se dão de acordo com o possível comportamento biológico destas neoplasias, ou seja,

independente do tipo histológico, geralmente elas são tratadas da mesma maneira, dependendo apenas do seu grau de agressividade.

Clinicamente pode-se dividi-los nos seguintes grupos (KEMPSON et al. 2001):

Quadro 1 - Classificação clínica.

Grupo	*Comportamento*	*Tratamento*
	Tumores clinicamente benignos	
Ia	Excisão local geramente é curativa. Nunca metastatiza	Excisão local
Ib	Recorrências locais podem ocorrer, mas não são destrutivas. Nunca metastatizam	Excisão local da lesão primária e das recorrências se houverem
	Tumores de agressividade intermediária	
IIa	Recorrências locais são comuns e podem ser destrutivas. Nunca metastatizam	Excisão local com margens ampliadas
IIb	Recorrências locais são comuns. Metástases podem ocorrer se sofrerem desdiferenciação	Excisão local com margens ampliadas. Às vezes podem ser utilizadas terapias adjuvantes
	Tumores clinicamente malignos (sarcomas)	
IIc	Recorrências locais são comuns. Metástases raramente ocorrem.	Excisão local com margens ampliadas. Às vezes podem ser utilizadas terapias adjuvantes
III	Recorrências locais são comuns. Podem metastatizar	Excisão local com margens ampliadas. Terapias adjuvantes
IV	Doença sistêmica	Terapia adjuvante, neoadjuvante, cirurgia

1.1.2 Graduação dos sarcomas

Existem alguns sistemas de graduação de sarcomas. Dentre eles os 2 mais utilizados são o French Fédération Nationale des Centres de Lutte Contre le Câncer (FNCLCC) (GUILLOU et al. 1997) e o National Cancer Institute (NCI) (COSTA et al. 1984).

O NCI leva em consideração o tipo histológico, a celularidade, o pleomorfismo e o índice mitótico. Dá-se notas de 1 a 3 para cada categoria e depois soma-se os scores. O FNCLCC é o sistema mais utilizado em todo o mundo. Leva em consideração o grau de diferenciação, o índice mitótico e a porcentagem de necrose. A cada característica é dada uma pontuação, e depois soma-se todos os pontos obtidos por cada tumor. Quanto maior a pontuação maior é o grau histológico, ou seja, mais agressivo é o tumor. A graduação da FNCLCC pode ser vista no quadro abaixo:

Quadro 2 - Graduação da FNCLCC.

Diferenciação Tumoral	
Score 1	Sarcomas semelhantes ao tecido mesenquimal de origem
Score 2	Sarcomas onde é possível definir o tecido de origem
Score 3	Sarcomas indiferenciados, embrionários e de origem incerta
Índice Mitótico	
Score 1	0-9 mitoses por CGA
Score 2	10-19 mitoses por CGA
Score 3	>20 mitoses por CGA
Necrose	
Score 0	Ausência de necrose
Score 1	<50% necrose tumoral
Score 2	>50% necrose tumoral
Graduação Histológica Final	
Grau 1	Score total 2,3
Grau 2	Score total 4,5
Grau 3	Score total 6,7,8

Alguns tipos de sarcomas, independente das características descritas no Quadro 2, são considerados de alto grau por definição. Tal fato é devido à evolução clínica destes tumores, que invariavelmente são agressivos com prognóstico reservado. Para estes tipos de sarcomas, não se aplica nenhuma graduação, como é o caso, por exemplo, dos sarcomas sinoviais e dos sarcomas pleomórficos.

1.2 ASPECTOS MOLECULARES E SARCOMAS

Os sarcomas representam um grupo bastante heterogêneo que abrange mais de 50 tipos histológicos. Com o advento dos estudos moleculares, foi possível melhor caracterizar alguns destes tumores.

Geneticamente, os sarcomas podem ser divididos em dois grandes grupos (MELTZER e HELMAN 2003). O primeiro é constituído por aqueles tumores que possuem uma alteração genética específica tais como translocações cromossômicas e mutações ativadoras que parece ser a causa central da patogênese. Deste grupo fazem parte o sarcoma sinovial, dermatofibrossarcoma protuberans, sarcomas alveolar de partes moles, GISTs (“gastrointestinal stromal tumors”) entre outros. O segundo grupo de sarcomas não apresenta alterações moleculares específicas, mas sim uma complexa desorganização cariotípica com graves alterações genéticas e instabilidades cromossômicas. Neste grupo estão incluídos os lipossarcomas, leiomiossarcomas, sarcomas pleomórficos entre outros.

Dentre o grupo com alterações genéticas específicas, é possível se classificar os sarcomas de acordo com a sua mutação específica. A presença da fusão gênica SYT-SSX resultante da translocação X;18 por exemplo, é patognomônica do sarcoma sinovial. Outros exemplos podem ser vistos no Quadro 3. A maioria destas translocações produz fatores de transcrições quiméricos, capazes de desregular a expressão de inúmeros genes alvos (MAY et al. 1993).

Nos rabdomiossarcomas alveolares, por exemplo, há uma fusão entre o gene *PAX3* localizado no cromossomo 2 com o gene *FKHR* localizado no cromossomo 13, resultando em um gene de fusão *PAX3-FKHR* que atua como fator de transcrição, levando a uma desregulação da expressão gênica (GALILI et al. 1993; BARR et al. 1998).

No caso dos GISTs, uma mutação somática no gene *KIT*, leva a uma ativação constitutiva de receptor tirosino-kinase que por sua vez resulta em alterações na proliferação celular e tumorigênese (BERMAN e O'LEARY 2001; LONGLEY et al. 2001; MIETTINEN et al. 2001).

Quadro 3 - Exemplos de sarcomas com translocação cromossômica específica.

Tumor	**Translocação**	**Produto de fusão**
Sarcoma de Ewing	t(11;22), t(21;22),	*EWS-FLI1*
	t(7:22), t(1;16)	*EWS-ERG*
Sarcoma de células claras	t(12;22)	*EWS-ATF1*
DSRCT*	t(11;22)(p13;q12)	*EWS-WT1*
Lipossarcoma de células	t(12;16)	*TLS-CHOP*
redondas		*EWS-CHOP*
Rabdiomiossarcoma alveolar	t(2;13); t(1;13)	*PAX3-FKHR*
Sarcoma sinovial	t(X;18)	*SYT-SSX1*
		SYT-SSX2
Sarcoma Alveolar de partes	t(X;17)	*TFE3-ASPL*
moles		
Lipossarcoma Mixóide	t(12;16), t(12;22)	*TLS-CHOP*
		EWS-CHOP

* DSRCT: Desmoplastic small round cell tumor

Naqueles sarcomas sem alterações moleculares específicas, os mecanismos genéticos alterados identificados, geralmente incluem distúrbios

em genes do ciclo celular, tal como o gene *TP53,* o gene *INK4* e o gene *RB1* (DEI et al. 1996; STRATTON et al. 1999). Alguns trabalhos experimentais demonstraram que camundongos com defeitos em *TP53* ou *INK4*, desenvolvem sarcomas. (DONEHOWER et al. 1992; HARVEY et al. 1993; NAKANISHI et al. 1998; KAMIJO et al. 1999). Entretanto, como a maioria dos pacientes não possue mutações germinativas destes genes, não se sabe quando estas mutações ocorrem durante a tumorigênese, ou quais outras mutações são necessárias para o seu desenvolvimento. Acredita-se que defeitos nas vias de reparo possam estar presentes assim como alterações em outros genes que ajudem a manter a estabilidade cromossômica.

Encontramos características distintas entre os dois grupos de sarcomas, como podemos ver no Quadro 4 citado por BORDEN et al. (2003).

Quadro 4 - Diferenças entre sarcomas com e sem alterações genéticas específicas.

Característica	Sarcomas com alterações genéticas conhecidas	Sarcomas sem alterações genéticas conhecidas
Cariótipo	simples	complexo
Translocações	Recíprocas e específicas	Não recíprocas e Inespecíficas
Média de idade	27 anos	57 anos
Alterações em p53	Baixa	Alta
Impacto das alterações p53	Forte	Fraca
Incidência em Síndromes RB e Li-fraumeni	Raro	Comum
Incidência entre sarcomas rádio-induzidos	Rara	Comum

1.3 EXPRESSAO GÊNICA

A grande diversidade de tumores, mesmo quando derivados dos mesmos tecidos, impõe um dos desafios centrais para o diagnóstico preciso e para o tratamento efetivo do câncer. Pacientes portadores de tumores semelhantes histopatologicamente podem apresentar diferentes evoluções clínicas e respostas terapêuticas. A dificuldade em classificar os tumores em grupos relativamente homogêneos é um obstáculo importante para o progresso do diagnóstico do câncer e da busca de terapias mais efetivas. Geralmente, uma mistura de critérios incluindo diagnóstico morfológico, medidas da extensão e da disseminação da doença e uma variedade de marcadores prognósticos é considerada para auxiliar nas condutas

terapêuticas. Como o fenótipo de cada tipo de câncer é o resultado de alterações em muitas vias gênicas diferentes, o uso de marcadores únicos significa uma simplificação excessiva da etiologia e progressão do câncer.

A transformação de um tecido normal em neoplásico é acompanhada por uma mudança do padrão de expressão de uma série de genes que se reflete em uma diferente oferta de RNA mensageiro (RNAm) correspondente a estes genes, incluindo a presença de transcritos provenientes de moléculas anormais de RNAm originárias dos genes alterados. Esta diferença na expressão gênica ocorre, não só entre tecido tumoral e não tumoral, mas também em diferentes fases evolutivas e entre variantes de um mesmo tumor. Logo, o perfil de expressão gênica pode não só fornecer informações para a compreensão do desenvolvimento tumoral como diferenças no nível de expressão entre tipos tumorais e tecido normal. Estas diferenças podem ser utilizadas como marcadores para diagnóstico ou prognóstico ou ainda servir de alvo para o desenvolvimento de novas drogas, uma vez que estes genes podem codificar antígenos tumorais específicos.

Com o aumento massivo da informação da seqüência do genoma humano, novas tecnologias como SAGE (serial analysis of gene expression) (VELCULESCU et al. 1995) e microarrays de cDNA (SCHENA et al. 1998) tem sido aplicadas à análise da expressão de milhares de genes em um único experimento, levando à identificação de genes importantes na tumorigênese. Os microarrays podem ser usados para medir a expressão de

milhares de genes simultaneamente, gerando um perfil de expressão do tecido estudado.

1.3.1 DNA MICROARRAY

O “DNA microarray” ou “biochip de DNA” é uma poderosa tecnologia que permite a análise simultânea da expressão de milhares de genes (RUSSO et al. 2003). O método consiste na hibridização das seqüências de cDNA ou oligonucleotídeos imobilizados em uma lâmina de vidro, com moléculas correspondentes a RNAm de células, tecidos ou outras fontes biológicas marcadas com corantes fluorescentes e detectadas por um *scanner* de fluorescência. O uso de diferentes corantes fluorescentes permite que o RNA mensageiro de diferentes populações celulares ou tecidos seja marcado com diferentes corantes, misturado e hibridizado na mesma lâmina de vidro, resultando em uma hibridização competitiva. Normalmente as hibridizações são feitas utilizando uma amostra referência (que pode ser *pool* de amostras normais, tumorais ou linhagens celulares), a qual é marcada com um corante fluorescente distinto da amostra teste. Durante a hibridização as seqüências marcadas com moléculas fluorescentes reagem com suas seqüências complementares ordenadas na lâmina de vidro. Depois da hibridização e lavagem a lâmina de vidro é submetida à varredura por laser usando os dois comprimentos de ondas correspondentes às duas moléculas fluorescentes usadas. Como a hibridização é competitiva, os valores de fluorescência obtidos revelam níveis relativos de expressão de cada transcrito na amostra teste comparada com a amostra referência. O uso da referência produz uma medida básica de expressão para cada gene, possibilitando a normalização e comparações de experimentos independentes (YANG et al. 2002). Após a normalização os

dados classicamente são submetidos a três análises matemáticas diferentes. A primeira visa buscar genes diferencialmente expressos entre as amostras estudadas. A segunda busca reconhecer padrões de expressão gênica nos diferentes casos. Hoje também é possível fazer um terceiro tipo de análise na qual se buscam classificadores, ou seja, um grupo pequeno de genes que possa separar dois estados sendo a idéia básica a introdução destes classificadores na prática clínica.

A primeira correlação clínica entre padrões de expressão gênica com evolução clínica foi mostrada por ALIZADEH et al. (2000), no estudo de linfoma difuso de grandes células B (LDGCB), que é clinicamente heterogêneo. Enquanto em torno de 60% dos pacientes sucumbem à doença, o restante dos pacientes tem boa resposta à terapêutica e apresentam sobrevida prolongada. Essa variabilidade na progressão da doença pôde ser correlacionada com o padrão de expressão gênica revelado por microarrays de cDNA, separando os distintos grupos de LDGCB.

A análise da expressão usando microarrays de cDNA pode também prover novos conhecimentos sobre a progressão do câncer. SGROI et al. (1999) compararam o padrão de expressão gênica de tumores invasivos e metástases. Entre 8.102 genes cuja expressão foi analisada, 90 genes foram identificados como apresentando alteração de pelo menos duas vezes entre os tumores invasivos e as metástases.

ONO et al. (2000) com o objetivo de identificar genes envolvidos na progressão do câncer de ovário, compararam o padrão de expressão de

9121 genes entre carcinomas de ovários e os respectivos tecidos normais. Cinqüenta e cinco genes foram identificados com aumento de expressão e 48 com diminuição de expressão nos tecidos tumorais. Além disso, 115 genes foram identificados como apresentando expressão diferencial entre adenocarcinomas serosos e mucinosos.

Recentemente, SEGAL et al. (2004) descreveram uma metodologia de análise de *microarray* que permite a identificação em um painel de amostras, da ativação ou inibição de um conjunto de genes relacionados a processos biológicos, como por exemplo, o controle do ciclo celular, apoptose, matabolismos de proteínas entre outros. Uma determinada condição biológica ou uma doença poderia ser então, caracterizada pela combinação da ativação ou inibição desses módulos.

1.3.2 Expressão gênica e Sarcomas

A aplicação de técnicas como cDNA microarray, oligo arrays e CGH (do inglês “copy genomic hybridization”), trouxeram muitas informações importantes para melhor conhecimento da gênese e comportamento das neoplasias mesenquimais.

A experiência mostrou ser possível separar alguns grupos de tumores mesenquimais de acordo com o seu perfil de expressão gênica como demonstrado por ALLANDER et al. (2002) que identificaram genes capazes de distinguir sarcomas sinoviais de sarcomas pleomórficos e fibrossarcomas e ainda genes capazes de separar os sarcomas sinoviais bifásicos dos monofásicos. NIELSEN et al. (2002) também demonstraram que GISTs,

sarcomas sinoviais, tumores neurais e um subgrupo de leiomiossarcomas, apresentavam um padrão distinto de expressão gênica, enquanto sarcomas pleomórficos, lipossarcomas e parte dos leiomiossarcomas, não apresentavam padrões específicos. Reforçando ainda mais estes resultados, SEGAL et al. (2003) notaram que sarcomas sinoviais, sarcomas de células claras, lipossarcomas mixoides e GISTs, também apresentavam assinatura molecular distinta enquanto sarcomas pleomórficos, fibrossarcomas e leiomiossarcomas, não apresentavam características gênicas distintas.

É interessante observar, que aqueles tumores que possuem alterações genéticas específicas já estabelecidas como translocações cromossômicas e “activing mutations”, são os mesmos que possuem perfis de expressão gênica distintos. Aqueles sarcomas que não possuem alterações específicas, mais uma vez se mostraram desorganizados do ponto de vista de expressão gênica.

Outra abordagem utilizada através de dados de microarray é a tentativa de se determinar a origem e a diferenciação celular de sarcomas com características histológicas que não permitem melhor caracterização quanto à sua histogênese como, por exemplo, os sarcomas sinoviais e sarcomas pleomórficos. NAGAYAMA et al. (2002) compararam o perfil de expressão gênica de sarcomas sinoviais com o perfil de diversos outros tipos histológicos de sarcomas e perceberam semelhanças de expressão gênica entre eles e os tumores malignos da bainha neural periférica (TMBNP) sugerindo uma origem neural para esta neoplasia.

Existem poucos trabalhos na literatura abordando dados de expressão gênica em sarcomas em relação à biologia tumoral e evolução clínica do paciente. REN et al. (2003) encontraram um grupo de genes capazes de discriminar os leiomiossarcomas de outros tipos histológicos de sarcomas e identificaram genes capazes de discriminar grupos relacionados ao grau histológico, e comportamento clínico em 11 leiomiossarcomas estudados.

Em 2005, BAIRD et al. publicaram a maior série de tumores mesenquimais estudadas através de cDNA microarrays. Os resultados obtidos dos 181 casos estudados, só vieram a confirmar os achados já publicados anteriormente onde alguns tipos histológicos de sarcomas têm perfis de expressão gênica estabelecidos e outros não. O estudo gerou enormes listas de genes diferencialmente expressos em cada grupo. Compreender a importância destes genes, suas reais funções e papéis no desenvolvimento destes tumores, é fundamental para aprimorar o tratamento, melhorando a sobrevida destes pacientes.

2 JUSTIFICATIVA

Muitas dúvidas ainda persistem sobre a origem, desenvolvimento e comportamento dos tumores de partes moles. São tumores de alta morbidade e mortalidade, ainda com poucas opções terapêuticas. Os estudos já realizados forneceram inúmeras informações a respeito de diferenciação celular e origem destas neoplasias, entretanto ainda há uma serie de indagações e perguntas a serem respondidas em relação à biologia tumoral e comportamento destas neoplasias.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Buscar genes diferencialmente expressos nos diversos tipos de tumores de partes moles.

3.2 OBJETIVOS ESPECÍFICOS

- Determinar e comparar o perfil de expressão gênica de amostras de tumores de partes moles de comportamento biológico distinto.
- Isolar genes responsáveis por características biológicas distintas nos tumores de partes moles como, por exemplo, genes responsáveis pela agressividade local e genes determinantes de metástases.
- Determinar e comparar o perfil de expressão gênica de amostras de tumores de partes moles de subtipos histológicos distintos

4 MATERIAL E MÉTODOS E RESULTADOS

Cada análise realizada até o momento foi motivo de manuscritos distintos. Os materiais e métodos e resultados deste trabalho serão apresentados, portanto, em forma de anexos que se encontram no final desta publicação. A seguir, uma breve discussão sobre os manuscritos anexados.

5 CONSIDERAÇÕES FINAIS

5.1 AGRESSIVIDADE LOCAL E POTENCIAL METASTÁTICO EM SARCOMAS (Anexo 1)

Os tumores de partes moles são raros, de alta morbidade e mortalidade dependendo do subtipo histológico. Os eventos moleculares relacionados aos seus comportamentos como agressividade local e metástase permanecem desconhecidos. A compreensão destes mecanismos é fundamental para discriminar aqueles pacientes que necessitam de tratamento sistêmico daqueles que possam ser tratados somente com abordagem local. A descoberta de novos genes e suas respectivas funções também é importante para o desenvolvimento de drogas mais efetivas. Neste estudo nós comparamos o perfil da expressão gênica de 102 amostras de tumores mesenquimais de diversos comportamentos biológicos.

Nós identificamos um grupo de seis genes relacionados funcionalmente à agressividade local (*SNRPD3, MEGF9, SPTAN-1, AFAP1L2, ENDOD1 e SERPIN5*), e outro grupo de seis genes relacionados a potencial metastático (*ZWINTAS, TOP2A, UBE2C, ABCF1, MCM2 e ARL6IP5*). Como recentemente revisto por CHIANG e MASSAGUÉ (2008), as células cancerosas acumulam alterações responsáveis pelas diferentes etapas na progressão tumoral. Alguns tumores possuem alterações capazes

de promover a expansão do tumor localmente, mas não são suficientes para o desenvolvimento de metástases. Geralmente, a iniciação do processo metastático requer alterações nos genes relacionados à invasão local (motilidade e remodelamento da matriz extracelular), angiogênese e transição epitélio-mesênquima. Para o estabelecimento da metástase, os genes relacionados à angiogênese e resposta imune e associados a alterações órgão-específica necessitam estar alterados. O set de genes que nós identificamos parece seguir este racional. *SERPIN5, SPTAN1 e MEGF9* estão envolvidos em invasão, destruição tecidual e motilidade celular, enquanto *UBE2C, ARL6IP5, MCM2, TOP2A e ABCF1* estão envolvidos no aumento do metabolismo, migração celular, ciclo celular, proliferação celular e transformação maligna.

As fibromatoses do tipo desmóide são exemplos típicos de tumores mesenquimais que mostram a distinção de duas etapas. São tumores bastante agressivos localmente, mas sem potencial metastático. A célula tumoral não teria capacidade de invadir vasos sanguíneos, sobreviver no sistema circulatório ou se implantar em outros sítios? De acordo com nossos resultados vimos que as fibromatoses têm uma hipoexpressão de genes envolvidos em aumento do metabolismo e proliferação celular, em relação aos sarcomas, o que pode ser uma explicação ao não desenvolvimento de metástase. Quando comparamos genes comuns aos dois grupos (fibromatoses e sarcomas), mas diferencialmente expressos em tumores mesenquimais benignos (sem potencial de invasão local e metástase), vimos que não há diferença de expressão entre alguns genes,

dentre eles, genes relacionados à degradação de matriz extracelular e destruição tecidual.

Uma característica distinta aos sarcomas em relação aos carcinomas e melanomas é o seu potencial metastático quase exclusivo à via hematogênica. Ao contrário dos carcinomas, sarcomas, mesmo aqueles com alto potencial metastático, raramente acometem linfonodos. Quando isto ocorre geralmente é restrito a tipos histológicos específicos que possuem células de formato epitelióide como o sarcoma epitelióide ou possuem capacidade de diferenciação epitelial verdadeira como é o caso dos sarcomas sinoviais.

Tal fato sugere que para o estabelecimento de metástase não basta haver alterações só em grupos de genes responsáveis por invasão, remodelamento tecidual, angiogênese, resposta imune e associados a alterações órgão-específica como proposto por CHIANG e MASSAGUÉ 2008. Parece que diferenças de expressão nos genes relacionados à diferenciação celular, responsáveis por alterações fenotípicas das células, também devem ser determinantes para o estabelecimento de metástases em sítios específicos.

5.2 ASSINATURA MOLECULAR E SARCOMAS (Anexos 2 e 3)

O reconhecimento de assinaturas moleculares trouxe melhor entendimento da patogênese destas neoplasias. A experiência mostrou ser

possível separar alguns grupos de tumores mesenquimais de acordo com o seu perfil de expressão gênica.

Através de análise não supervisionada, o perfil de expressão gênica global dos casos analisados, foi capaz de diferenciar três grandes grupos de tumores de parte moles: fibromatoses (FM), sarcomas sinoviais (SS) e os demais tipos (Figura 1 do Anexo 2)

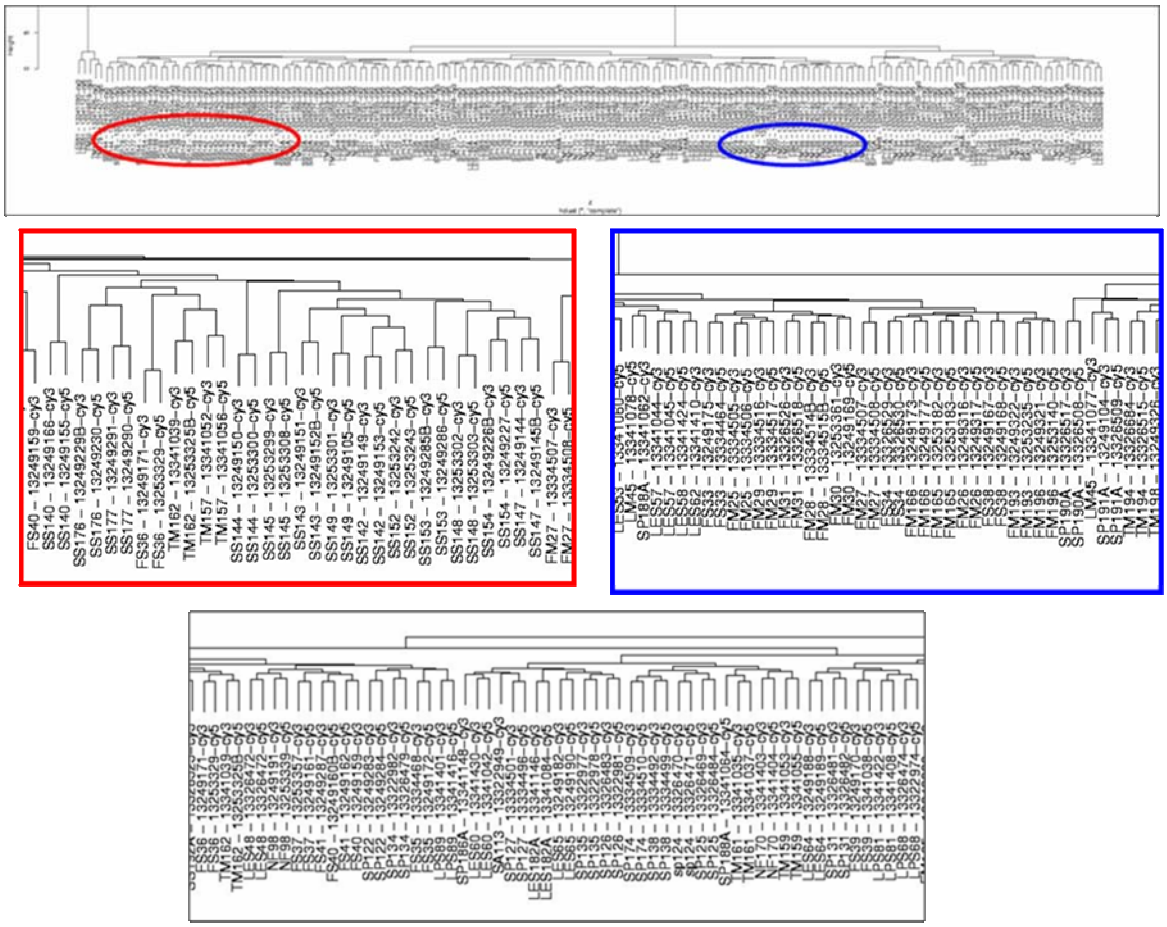

Figura 1 - Dendograma mostrando a distribuição das amostras, quando analisados todos os genes.

Os sarcomas sinoviais são raros, compreendendo aproximadamente 5 a 10% dos sarcomas de partes moles (KRANSDORF 1995). São mais comuns em extremidades, embora possam originar-se em qualquer parte do corpo sem qualquer relação com membranas sinoviais (FISHER 1990). Histologicamente podem ser subclassificados em monofásicos e bifásicos, baseados na presença ou ausência de diferenciação glandular epitelial junto a grupos de células tumorais fusiformes (MIETTINEN et al. 1983; MIETTINEN e VIRTANEN 1984). Esses tumores fazem parte do grupo de sarcomas com alteração molecular específica, apresentando em mais de 90% dos casos, a translocação t(X;18)(p11; q11) e auxiliando no diagnóstico de casos não característicos (The Cancer Genome Anatomy Project- CGAP 2002).

Dos genes diferencialmente expressos encontrados entre sarcomas sinovias e demais grupos, os mais significativos foram *TLE1, EFNB3, COL9A3, CHAF1A e SUHW2*. O gene *TLE1* já foi reportado por diversos outros estudos de cDNA microarray, realizados por grupos independentes (BAIRD et al. 2005, NIELSEN et al. 2005; TERRY et al. 2007). Ele tem sido considerado um excelente discriminante entre sarcomas sinoviais e outros sarcomas, incluindo aqueles com morfologia semelhante como tumores malignos da bainha do nervo periférico e fibrossarcomas. Proteínas TLE1 são co-repressores transcricionais que inibem vias de sinalização como, por exemplo, WNT. Tal fato é corroborado com a morfologia do sarcoma sinovial a qual não conseguimos relacionar a um tecido diferenciado presente no organismo humano. Anticorpo anti TLE-1 já foi validado em uma série de

sarcomas por TERRY et al. (2007) e se mostrou um marcador imunoistoquímico capaz de auxiliar no diagnóstico do sarcoma sinovial.

Na procura de classificadores pelo discriminador linear de Fisher, onde realizamos uma busca exaustiva com genes pertencentes a uma mesma via metabólica, encontramos 3 trios de genes capazes de separar os sarcomas sinoviais com 100% de acerto e outros 70 trios de genes com 99% de acerto de todos os outros tipos histológicos estudados.

Além do *TLE1*, conseguimos validar alguns destes genes encontrados através de Q-PCR e imunoistoquímica. Tais achados foram motivos de trabalhos distintos já mostrados em congressos nacionais e internacionais e estão sendo preparados manuscritos distintos. Maiores detalhes podem ser encontrados nos Anexos 2 e 3.

Outro achado significativo do estudo de expressão gênica foi a respeito das fibromatoses. As fibromatoses músculo aponeuróticas, também chamadas de tumores desmóides, apresentam um comportamento biológico muito peculiar. São extremamente agressivas localmente, capazes de infiltrar tecidos vizinhos, inclusive tecidos ósseos, mas não possuem potencial metastático. Tal característica nos levou ao desenvolvimento do primeiro manuscrito apresentado como anexo 1 nesta tese.

As fibromatoses mostraram ser um grupo bastante coeso e também com perfil de expressão gênica característico, capazes de distinguí-las de outros tumores mesenquimais, tanto malignos como benignos. Este grupo se mostrou tão distinto que fomos capazes de encontrar 92 trios e 92 pares de genes capazes de separá-las com 100% dos fibrossarcomas e 3 trios

capazes de separá-las com 99% de acerto de todos os outros tumores avaliados.

SKUBITZ et al. (2004 a e b) compararam o perfil de expressão gênica de 12 fibromatoses com tecido muscular esquelético normal e encontraram 170 genes capazes de distinguí-las do tecido normal. Dos genes encontrados os mais relevantes foram *ADAM12, WISP-1, SOX-11 e FAPα*. Comparadas a outros tumores mesenquimais, observou a hiperexpressão de *COLAGENS I, V, XI, XII e VI-α2, SPONDIN-1, ADLICAN, FIBROMODULIN, TGF-β3 e ADAM12* nas fibromatoses. No nosso estudo os principais genes diferencialmente expressos entre fibromatoses e fibrossarcomas foram *SNAP25, NTPD7, MYO5A, TMEM66* e *USP1* e entre fibromatoses e demais tumores foram *ENTPD7,RNF167,SETD7,FUCA1 e VPS39.*

5.3 GFAP E SARCOMAS (Anexo 4)

A proteína glial fibrilar ácida (GFAP) é um membro da superfamília das proteínas de filamentos intermediários, com 51-kD, sendo o maior componente dos astrócitos, células ependimárias e células de Müller da retina, não sendo tipicamente expressa em oligodendrócitos maduros (DELELLIS e SHIN 2006). Em tecidos não gliais, a proteína GFAP está também presente nas células de Schwann, de Küpffer, células de sustentação da hipófise anterior e paraganglionares (DELELLIS e SHIN 2006). Há descrições na literatura de marcação imunoistoquímica para GFAP em osteócitos de ossos normais e condrócitos de epiglote, cartilagem

costal, cartilagem brônquica e traquéia (KEPES e PERENTES 1988; KASANTIKUL e SHUANGSHOTI 1989, DOLMAN 1989; VIALE et al. 1998; (DELELLIS e SHIN 2006). A expressão imunoistoquímica de GFAP tem sido relatada como útil no diagnóstico de mioepiteliomas (HORNICK e FLETCHER 2003). No entanto, a expressão de GFAP em tumores mesenquimais é pouco estudada. Esse desconhecimento sobre o assunto pode causar confusão diagnóstica. No último ano, em nosso serviço, tivemos dois casos de tumores de cabeça e pescoço (um de mandíbula e outro de palato) cujas biópsias eram formadas por neoplasias de padrão condro-mixóide e que baseados na expressão de GFAP, entre outros marcadores, foram diagnosticadas como prováveis mioepiteliomas. Após ressecção cirúrgica das lesões e análise de toda a peça, constatamos que se tratavam de osteossarcomas condroblásticos. Tal fato despertou-nos para a pesquisa da real expressão de GFAP nos diversos tumores mesenquimais.

Avaliamos através das técnicas de cDNA microarray, Q-PCR e imunoistoquímica, a expressão de GFAP em diversos tipos histológicos de tumores mesenquimais. Os achados encontrados nos mostraram que a expressão de GFAP é decorrente da capacidade de diferenciação cartilaginosa presente em alguns sarcomas.

Apesar da expressão imunoistoquímica da proteína GFAP ser utilizada em tumores mesenquimais no diagnóstico de mioepitelioma, este estudo vêm desmistificar o uso focal e quase específico do GFAP nesta neoplasia, evidenciando a importante consideração de outros diferenciais, principalmente tumores com diferenciação cartilaginosa, com destaque aos

osteossarcomas condroblásticos. Essa expressão provavelmente está relacionada à diferenciação cartilaginosa e não à célula mioepitelial, uma vez que estes tumores têm este potencial de diferenciação, assim como adenomas pleomórficos de glândulas salivares, cordomas cartilaginosos, hamartomas cartilaginosos do trato respiratório e siringomas condróides da pele, lesões que também podem expressar GFAP (KEPES e PERENTES 1988; DOLMAN 1989; KASANTIKUL e SHUANGSHOTI 1989; NOTOHARA et al. 1990).

6 PERSPECTIVAS FUTURAS

Os resultados gerados deste trabalho são motivos de novas teses que tem como objetivos a validação dos achados tanto ”in vitro” como “in vivo”, assim como a procura de novos genes a partir de novas perguntas biológicas. Os dados clínicos relativos a todos os pacientes do estudo estão coletados e disponíveis para estudos futuros que incluam perguntas clínicas e de respostas a tratamento.

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Anexo 1

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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.transoc.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 PFFE 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 MPNSTs, 6 GISTs, 6 neurofibromas, 4 alveolar soft part sarcomas, 4 leiomyomas, and 4 schwannomas, retrieved from archived samples.

Extraction, Amplification, and Labeling of the aRNA

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).

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). 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). Primer pairs for real-time polymerase chain reaction (PCR) were determined with the aid of Primer Express software 3.0 (Applied Biosciences) 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) 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 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) 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, 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 file for further statistical analysis. The software Prism 5 for Windows, version 5.02, 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 FDR [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.

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

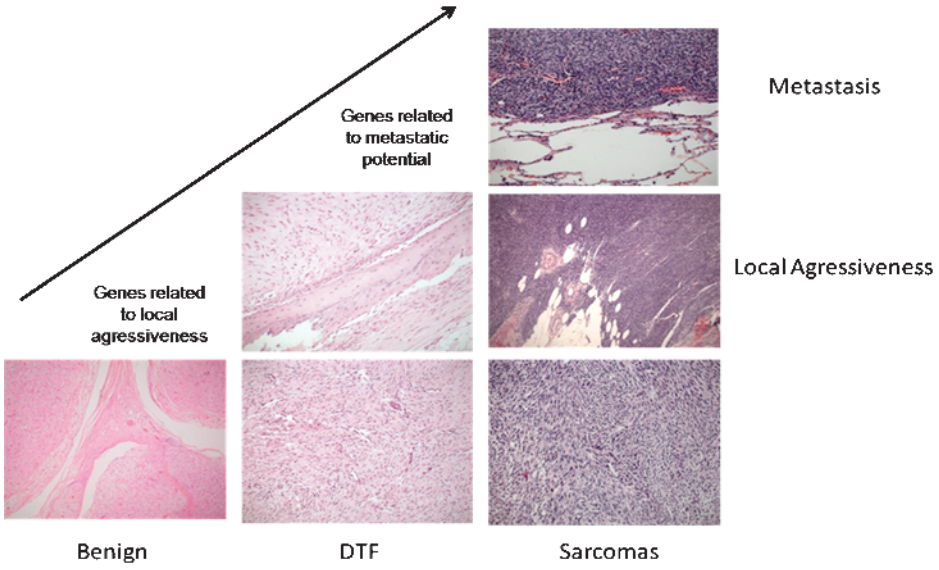

Figure 1. Histopathologic representation of diverse biological behavior in mesenchymal tumors (H&E staining).

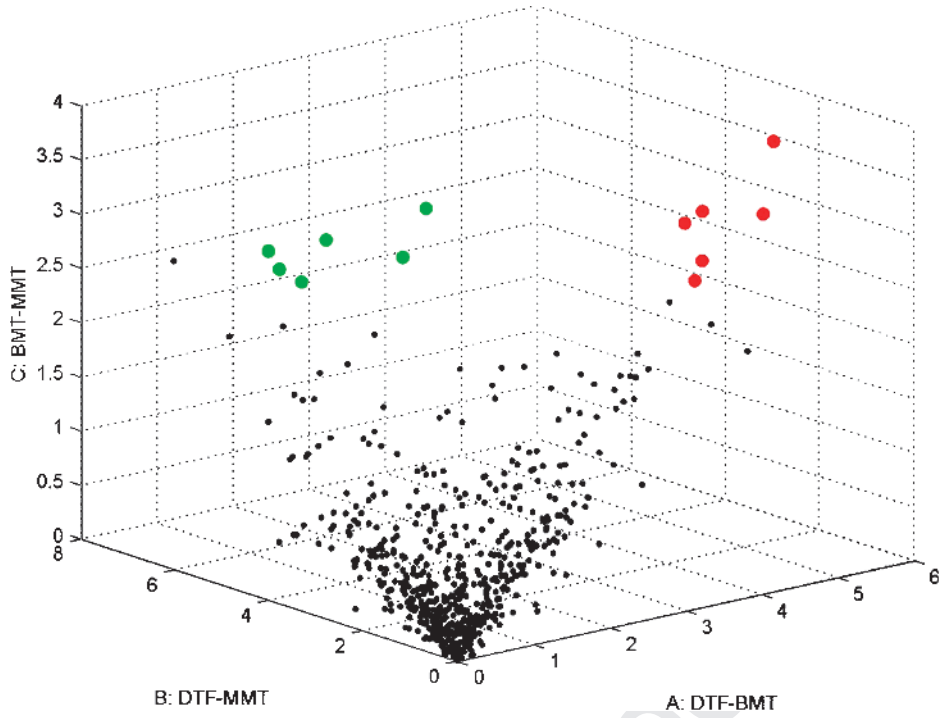

Figure 2. Three-dimensional scatter plot representing the expression of genes associated to local aggressiveness and metastasis. For each gene, we determined their nominal *P* values (Wilcoxon test, $-\log_{10}$) and plotted in a three-dimensional scatter plot. In red are the genes associated to local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

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, MPNST, 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 group when compared to the MMT group ($P < .0001$). Conversely,

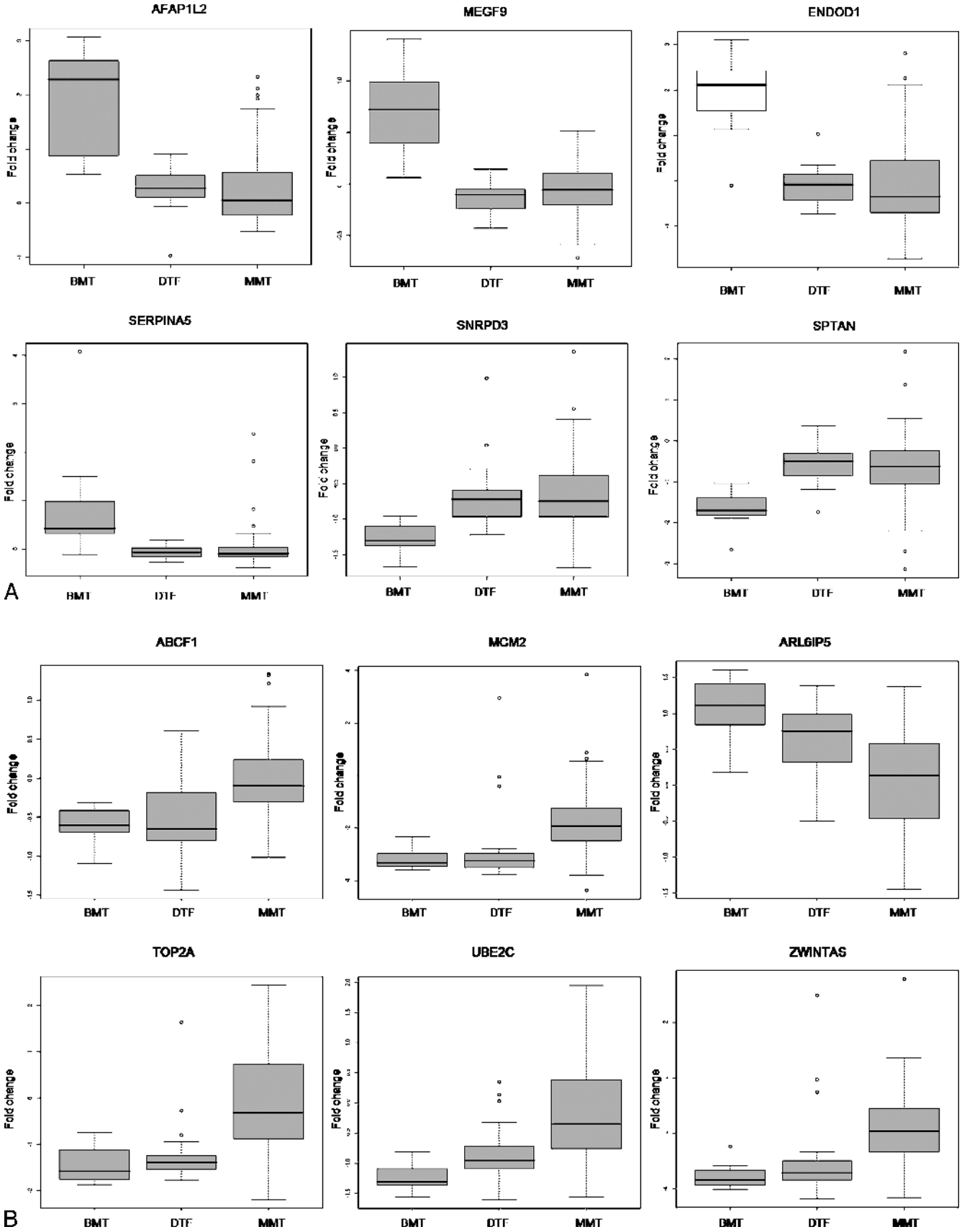

Figure 3. Representative box plot showing the expression (fold change) of the six genes related to local aggressiveness (*AFAP1L2*, *MEGF9*, *ENDOD1*, *SERPIN5*, *SNRPD3*, and *SPTAN*; A) and the six genes related to metastatic potential (*ABCF1*, *MCM2*, *ARL6IP5*, *TOP2A*, *UBE2C*, and *ZWINTAS*; B)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

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

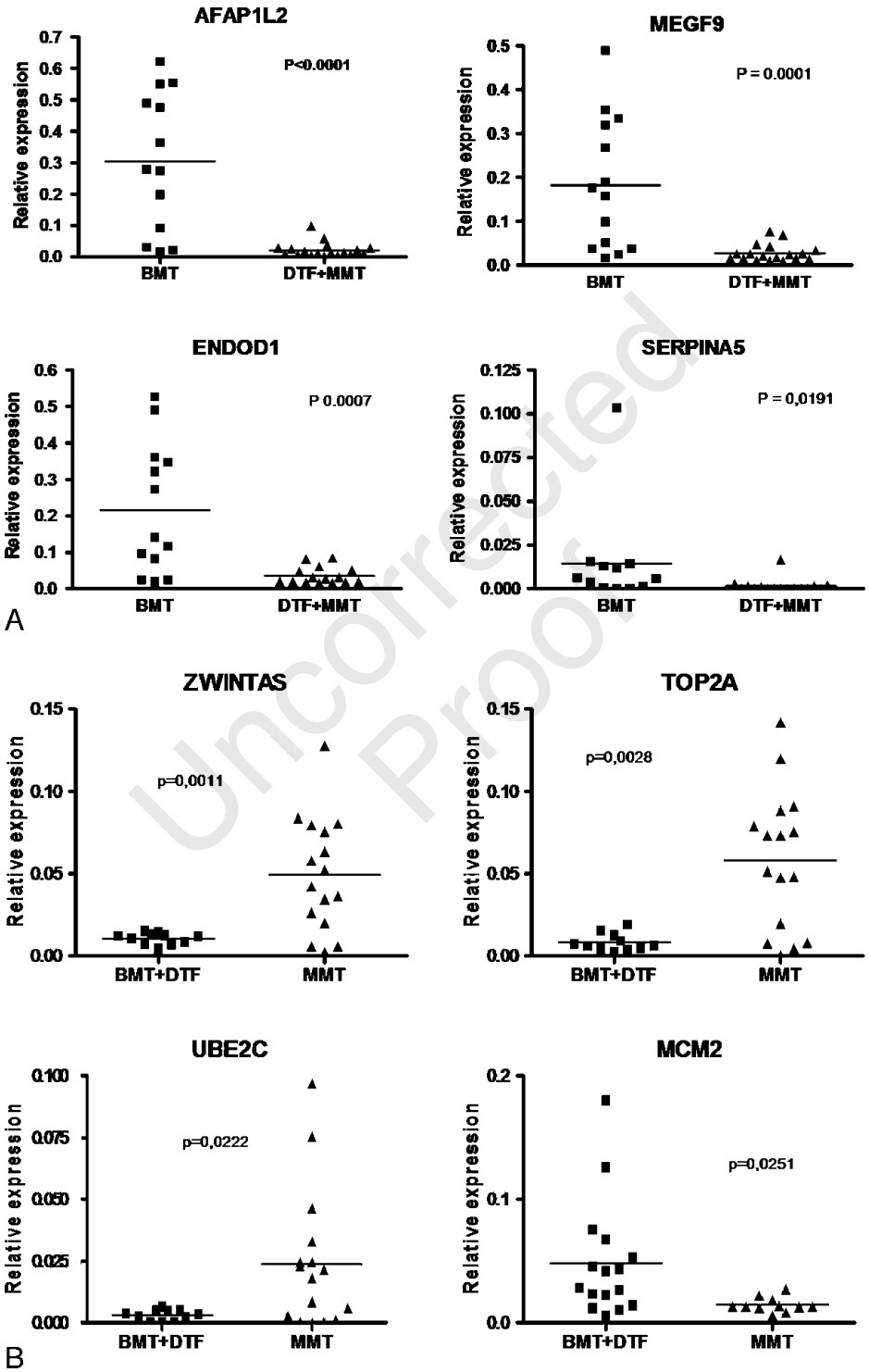

Figure 4. Validation by Q-PCR of the genes related to local aggressiveness (A) and metastasis (B). Total RNA used for Q-PCR was performed using SYBR green. *P* values were calculated by Mann-Whitney.

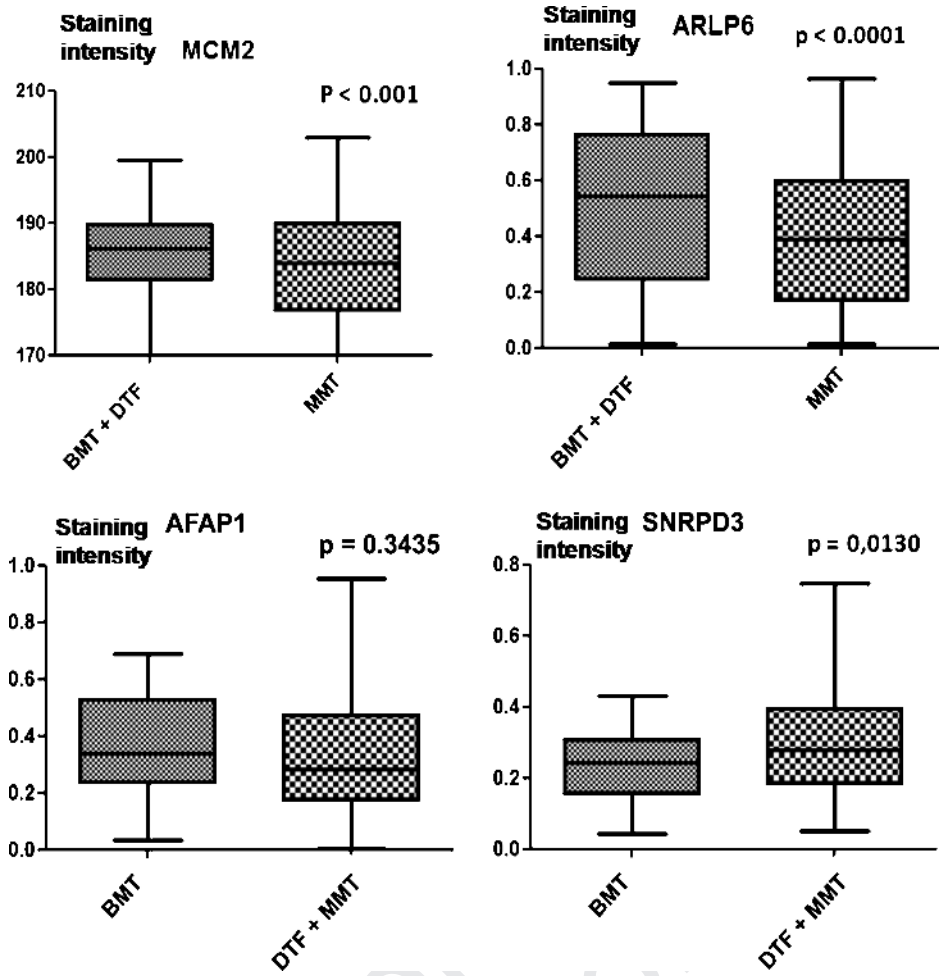

Figure 5. Representative box plot showing the protein expression of the two proteins related to local aggressiveness (AFAP1L2 and SNRPD3) and two proteins related to metastatic potential (MCM2 and ARL6IP5) in BMTs, DTFs, and MMTs.

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]. uPA and PAI-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

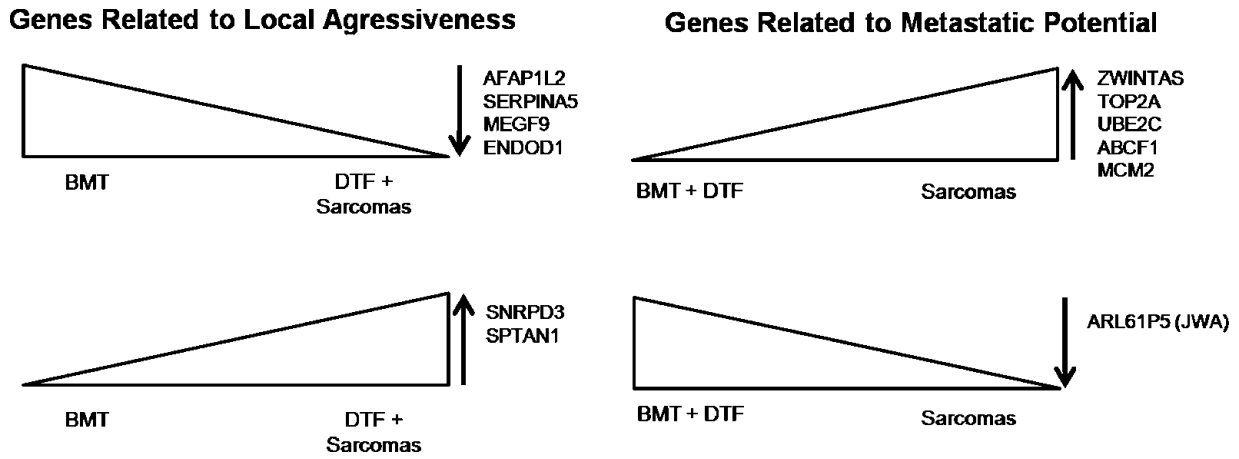

Figure 6. Schematic representation of selected genes based on a dichotomy in their expression profile related to local aggressiveness and metastatic potential in BMTs, DTFs, and MMTs.

corresponding nontumor tissue such as hepatocellular carcinoma and 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 MAPK 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/.

Q29

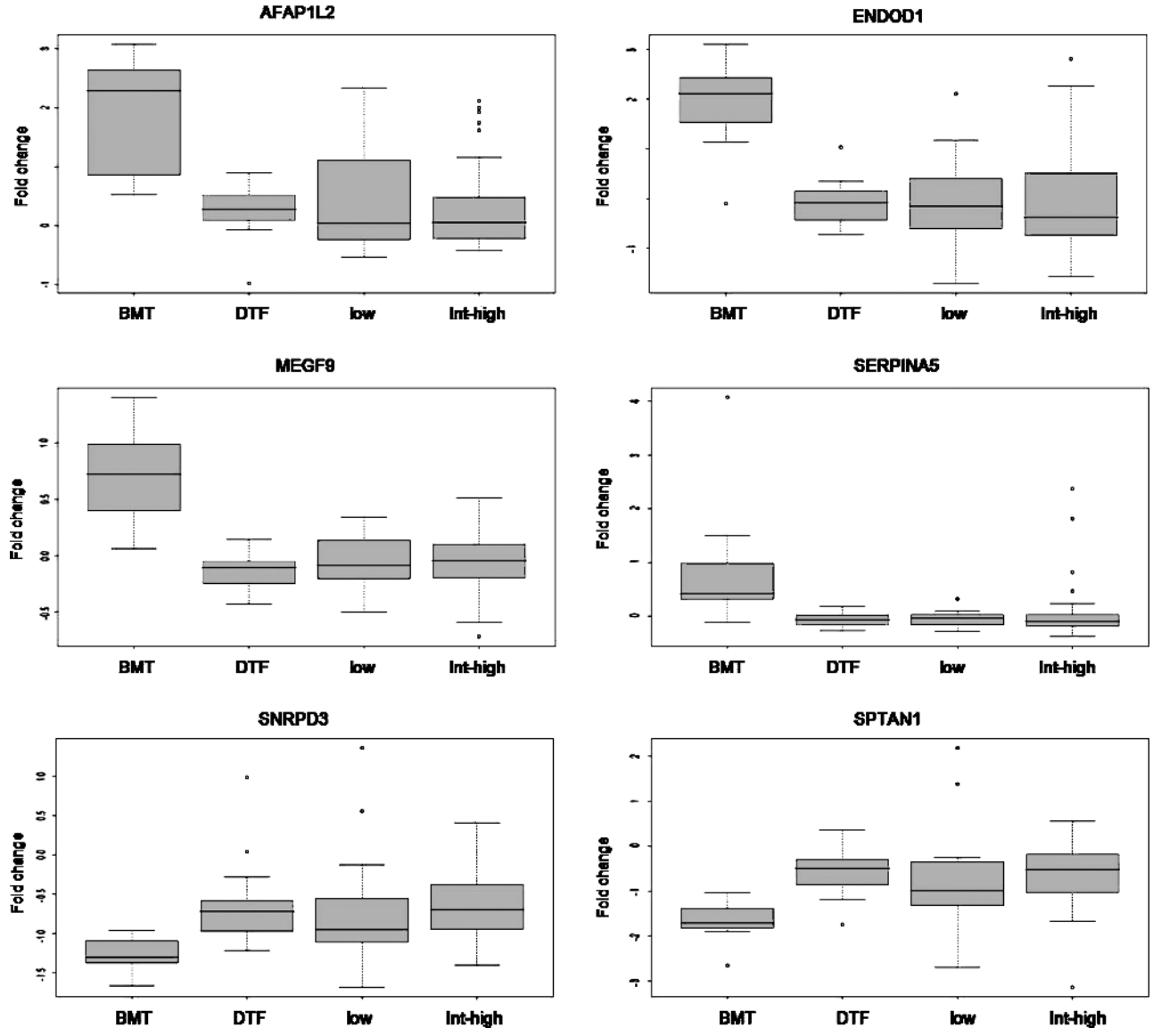

Figure W1. Representative box plot showing the expression (fold change) of the six genes related to local aggressiveness (*AFAP1L2*, *MEGF9*, *ENDOD1*, *SERPIN5*, *SNRPD3*, and *SPTAN*; A) and the six genes related to metastatic potential (*ABCF1*, *MCM2*, *ARL6IP5*, *TOP2A*, *UBE2C*, and *ZWINTAS*;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

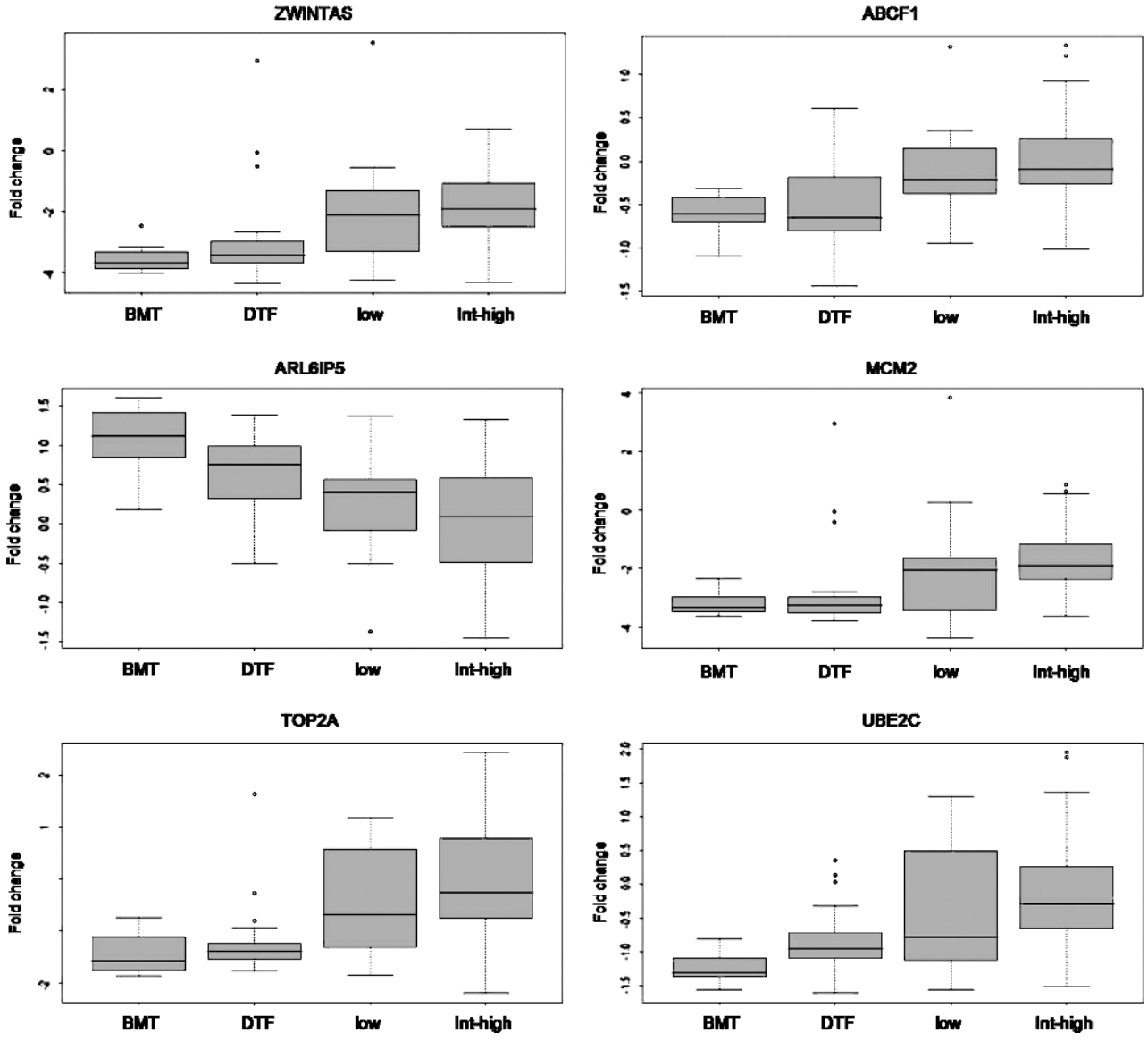

Figure W1. (*continued*).

Anexo 2

Trabalho apresentado no XXVI Congresso Brasileiro de Patologia, Bento Gonçalves RS, Novembro de 2007.

Prêmio SBP concedido aos 12 melhores trabalhos dentre os 736 submetidos ao congresso

CERTIFICADO

A Sociedade Brasileira de Patologia confere ao trabalho

O PERFIL DE EXPRESSÃO GÊNICA SEPARA OS DIVERSOS TIPOS DE SARCOMAS: ANÁLISE DE GENES DIFERENCIALMENTE EXPRESSOS EM TUMORES MESENQUIMAIS DE PARTES MOLES ATRAVÉS DA TÉCNICA DE CDNA MICROARRAY.

da autoria de

Cunha; IW -Carvalho; KC -Martins; WK -Marques; SM -Fahham; LV -Simões; AC -Cristo; EB -Neves; EJ -Soares; FA -Reis; LF

o **Prêmio SPB** concedido aos **12 Melhores Trabalhos** dentre os 736 submetidos ao XXVI CONGRESSO BRASILEIRO DE PATOLOGIA, realizado em Bento Gonçalves, RS, no período de 14 a 17 de novembro de 2007.

Luiz Antonio Rodrigues de Freitas
Presidente da SBP

Carlos Thadeu Schmidt Cerski
Presidente do Evento

Helenice Gobbi
Presidente da Comissão Científica

XXVI CONGRESSO BRASILEIRO DE PATOLOGIA
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Anexo 2 – Trabalho apresentado no XXVI CBP, Bento Gonçalves-RS.

O perfil de expressão gênica separa os diversos tipos de sarcomas: análise de genes diferencialmente expressos em tumores mesenquimais de partes moles através da técnica de cDNA microarray.

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Anexo 2 – Trabalho apresentado no XXVI CBP, Bento Gonçalves-RS.

Sumário

Introdução: Os sarcomas são neoplasias raras e possuem uma grande importância devido a sua alta morbidade e mortalidade. São classificados histologicamente de acordo com a diferenciação celular. O reconhecimento de assinaturas moleculares trouxe melhor entendimento da patogênese destas neoplasias. **Objetivos:** Avaliar o perfil de expressão gênica de tumores mesenquimais (TM), a fim de identificar genes relevantes ao diagnóstico, prognóstico e tratamento. **Material e Métodos**: O RNA de 102 casos de TM foi hibridizado em lâminas de vidro contendo seqüências imobilizadas de 4800 genes. O diagnóstico histológico foi confirmado por imunoistoquímica e/ou FISH. Os resultados da expressão gênica diferencial foram analisados para identificação de classificadores moleculares. **Resultados:** O perfil de expressão gênica dos casos analisados globalmente foi capaz de diferenciar três grandes grupos de TM: fibromatoses (FM), sarcomas sinovais (SS) e os demais tipos. Foram encontrados 92 trios e 92 pares de genes capazes de separar com 100% de acerto as FM dos fibrossarcomas. Dentre os genes mais diferencialmente expressos destacamos os SNAP25, NTPD7, MYO5A, TMEM66 e USP1. Os SS formaram um grupo distinto, sendo encontrados 3 trios de genes capazes de separá-los com 100% de acerto e outros 70 com 99% de acerto de todos os tipos histológicos. Os genes mais diferencialmente expressos foram TLE1, EFNB3, COL9A3, CHAF1A e SUHW2. As vias metabólicas analisadas não indicam expressão entre os grupos, apenas a via do BRAF parece discriminar as FM. **Conclusão:** A assinatura molecular dos TM indica diferenças importantes na separação dos grupos histológicos, podendo contribuir no diagnóstico e melhor entendimento de sua patogênese.

Apoio: FAPESP

Anexo 2 – Trabalho apresentado no XXVI CBP, Bento Gonçalves-RS.

1 INTRODUÇÃO

Tumores de partes moles são neoplasias raras correspondendo a menos que 10% de todas as neoplasias (1) Os tumores benignos são dez vezes mais freqüentes que os malignos. Os tumores de partes moles malignos (sarcomas) correspondem a menos de 1% de todos os cânceres, mas apesar da baixa incidência, possuem uma enorme importância devido a altas taxas de morbidade e mortalidade. (2) Sarcomas ocorrem em todas as idades, o que varia é o tipo histológico mais presente em cada faixa etária. Ocorrem por todo o corpo, com variações também decorrentes de subtipos histológicos.

A etiologia da grande maioria dos tumores de partes moles é desconhecida. Fatores como radiação, infecções virais, imunodeficiências parecem ter implicações em alguns tipos de sarcomas, mas a grande maioria parece se desenvolver sem fatores ambientais aparentes.

Atualmente, eles são divididos de acordo com o tecido normal a que o tumor mais se assemelha. (2). Apesar da tentativa de discriminar os diversos tipos histológicos, clinicamente as opções de tratamentos são restritas, e se dão de acordo com o provável comportamento biológico destas neoplasias, ou seja, independente do tipo histológico, elas são tratadas de acordo com o seu grau de agressividade.

O FNCLCC (French Fédération Nationale dês Centres de Lutte Contre Le Cancer) é um dos sistema de graduação em sarcomas e foi aplicado nos casos do presente estudo. Leva em consideração o grau de diferenciação tumoral, o índice mitótico e a porcentagem de necrose. Alguns

tipos de sarcomas, independente das características acima, são considerados de alto grau por definição. Tal fato é devido à evolução clínica destes tumores, que invariavelmente são agressivos.

Mais recentemente, com o advento de estudos moleculares, a melhor caracterização destas neoplasias vem se tornando possível. (2) Geneticamente, os sarcomas podem ser divididos em dois grandes grupos (3). Um grupo que possui uma alteração genética específica que parece ser a causa central da patogênese como translocações cromossômicas e mutações ativadores (KIT). Deste grupo fazem parte os sarcomas sinoviais, sarcomas alveolares de partes moles e GISTs, entre outros. O outro grupo de sarcomas não apresenta alterações genéticas específicas, e sim uma complexa desorganização cariotípica com graves alterações genéticas e instabilidades cromossômicas (Ex: Lipossarcomas, Leiomiossarcomas, Fibrossarcomas, Sarcomas pleomórficos).

Dentre o grupo com alterações genéticas específicas, é possível se classificar os sarcomas de acordo com a sua translocação cromossômica. A maioria destas translocações produz fatores de transcrições quiméricos, capazes de desregular a expressão de inúmeros genes alvos (4). Naqueles sarcomas sem alterações específicas, os mecanismos genéticos alterados identificados, geralmente incluem distúrbios em genes do ciclo celular como TP53, INK4 e RB1 (5,6,7,8,9). Outros causas também já conhecidas são defeitos nas vias de fatores de crescimento como em IGF1 (rabdo e leiomiossarcomas) (10,11,12,13), c-kit (GISTs) (14,15,16) e c-met (sarcomas sinoviais e rabdomiossarcomas) (17,18,19,20).

Anexo 2 – Trabalho apresentado no XXVI CBP, Bento Gonçalves-RS.

Estudos utilizando a técnica de cDNA microarray em sarcomas, tem sido feitos na tentativa de melhor entendimento destas neoplasias. (21, 22, 23, 24, 25).

DNA *microarray* ou *biochip de* DNA é uma tecnologia que permite a análise simultânea da expressão de milhares de genes (26). O método consiste na hibridização das seqüências de cDNA ou óligos imobilizados em uma lâmina de vidro, com moléculas correspondentes a RNAm de células, tecidos ou outras fontes biológicas marcadas com corantes fluorescentes e detectadas por um *scanner* de fluorescência. Este conjunto de informações é capaz de gerar um perfil de expressão único de cada tumor, que pode ser comparado com outros tumores permitindo o desenvolvimento de classificações moleculares mais acuradas das diferentes neoplasias.

Neste estudo avaliamos o perfil de expressão gênica através de cDNA microarray em 102 casos de tumores mesenquimais, na tentativa de discriminar os diversos tumores de acordo com sua histologia e comportamento biológico.

2 MATERIAL E MÉTODOS

Amostras e pacientes

Foram obtidas 102 amostras de 100 pacientes operados no Hospital A.C.Camargo no período de julho de 1997 a janeiro de 2005, e armazenadas no banco de tumores desta mesma instituição. Todos os pacientes do estudo assinaram um termo de consentimento pós-informado. Os casos

foram revistos e utilizado imunoistoquímica para classificação histológica. Os casos foram divididos em 6 grupos principais, de acordo com a diferenciação histogenética.

Grupo 1: Neoplasias de diferenciação fibroblástica; 24 casos sendo 19 fibromatoses (TFB) e 6 fibrossarcomas (TFM)

Grupo 2: Neoplasias de diferenciação neural periférica; 11 casos sendo 5 TMBNP (TNM), 3 neurofibromas (TNB) e 2 schwanomas (TNB)

Grupo 3: Neoplasias de diferenciação muscular; 26 casos sendo 19 leiomiossarcomas (TMM), 2 leiomiomas (TMB), 2 sarcomas alveolares de partes moles(TMM) e 2 GISTs (TMM)

- Os sarcomas alveolares de partes moles foram incluídos neste grupo, devido a grande discussão na literatura acerca destes tumores sobre uma possível origem muscular. Os GISTs também foram incluídos neste grupo por antigamente serem considerados de origem muscular, apesar de hoje já se saber que são originados da célula de Cajal. Ainda se discute se esta célula seria um “híbrido” entre a célula muscular e a célula neural.

Grupo 4: Neoplasias adipocíticas; 6 lipossarcomas (LPS)

Grupo 5: Sarcomas Sinoviais (SS); 19

Grupo 6: Sarcomas Pleomórficos (SP);16

Quando necessário, foi realizado semi-microdissecção nas amostras para eliminação de tecido contaminante e todas as amostras de tumor apresentavam pelo menos 70% de células tumorais. O material selecionado foi submetido à extração de RNA.

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Extração, Amplificação e marcação de RNA

A extração de RNA total foi realizada utilizando-se Trizol® (Invitrogen, USA). Após extração, o RNA total foi purificado através do MINIQIT® (Qiagen) e amplificado por um único ciclo, conforme descrito por nosso grupo (27). Como RNA referência, utilizamos um pool de RNAs derivados de linhagens celulares humanas. As amostras foram marcadas com os fluorocromos Alexa 555 e 647.

Hibridização e escaneamento do cDNA microarray:

As amostras foram hibridizadas em lâminas de vidro contendo sequências de 4800 cDNAs, preparadas em nosso laboratório(28). Pré-hibridização, hibridização e lavagem foram feitas como descrito anteriormente (29). As lâminas foram escaneadas utilizando-se um scanner à laser (ScanArray Express, Perkin-Elmer Life Sciences, Boston, MA) e os dados foram obtidos pelo software do ScanArray Express utilizando-se o método do histograma.

Análise estatística:

Para análise estatística, foi utilizado o programa R. Após correção do sinal de fundo local, os dados de cada lâmina foram normalizados pelo método de Lowes. Para identificação dos genes mais diferencialmente expressos, foi usado o teste não paramétrico de Mann-Whitney. Para análises de agrupamento, aplicamos um algorítimo de agrupamento não

supervisionado, baseado na distância euclidiana. Os genes foram ordenados com base na distância de correlação.

Classificadores:

Neste estudo também foi feita uma busca exaustiva por trios de genes classificadores capazes de distinguir os diversos tumores. Os trios de genes foram construídos pelo discriminador linear de Fisher. Este método tem sido o método mais utilizado em estudos de nosso grupo (30,31) e optamos por utilizar os resultados obtidos por este método.

RT- PCR

Para detecção da translocaçao SYT/SSX, o RNA total das amostras suspeitas foi transcrito reversamente e o cDNA utilizado como molde para PCR. O iniciador senso corresponde ao gene SYT e o iniciador antisenso corresponde ao gene SSX. O produto da reação foi fracionado em gel de agarose e corado com brometo de etídio.

3 RESULTADOS

O perfil de expressão gênica global dos casos analisados, sem nenhuma seleção de genes potencialmente alterados, foi capaz de diferenciar três grandes grupos de tumores de parte moles: fibromatoses (FM), sarcomas sinovais (SS) e os demais tipos. (figura 1) Um dado interessante que pudemos observar foi que três amostras, 1 fibrossarcoma

(FS36) e 2 Tumores malignos da bainha do nervo periférico (TMBNP) (TM157 e TM162) se agruparam junto a eles. Na revisão histológica destes casos, já havíamos levantando a possibilidade de se tratarem de sarcomas sinovias e não dos diagnósticos previamente emitidos. Como os SS apresentam uma translocação cromossômica específica envolvendo os cromossomos X e 18 (SSX-SYT), (32, 33) optamos por avaliar esta translocação através de RT-PCR em todos os casos de SS, nestes 3 casos que se agruparam junto a eles e em mais 2 casos que de acordo com a revisão histológica poderiam também corresponder a SS. A translocação estava presente em todos os casos de SS, e naqueles 3 casos que se agruparam junto a eles, o que nos confirmou que se tratavam realmente de SS. Os dois casos que poderiam corresponder a SS pela morfologia, mas que não se agruparam juntos, não possuíam a translocação e, portanto, não eram SS. O resultado da PCR pode ser observado na figura 2.

Os genes mais diferencialmente expressos entre sarcomas sinovias e demais grupos foram TLE1, EFNB3, COL9A3, CHAF1A e SUHW2. Na procura de classificadores pelo discriminador linear de Fisher, onde realizamos uma busca exaustiva com genes pertencentes a uma mesma via metabólica, foram encontrados 3 trios de genes capazes de separar os SS com 100% de acerto e outros 70 com 99% de acerto de todos os tipos histológicos. (figura 3C).

Analisando tumores com a mesma diferenciação celular, mas comportamentos biológicos distintos como fibromatoses e fibrossarcomas, vimos que os genes mais diferencialmente expressos entre eles foram

SNAP25, NTPD7, MYO5A, TMEM66 e USP1. Pudemos encontrar 92 trios e 92 pares de genes capazes de separá-los com 100% de acerto. (tabelas 2 e 3) (figura 3a)

Avaliamos diversas vias metabólicas na tentativa de estabelecer vias ativas e/ou inativas nos diversos grupos. A via do BRAF parece discriminar as FM sendo o gene FGFR1 mais expresso e o PDGFRL menos expresso nas FM em relação aos demais tumores estudados. (figura 4)

4 DISCUSSÃO

Alguns estudos com diversas abordagens têm demonstrado diferentes perfis de expressão gênica em sarcomas. Sarcomas sinoviais, GISTs, e lipossarcomas mixóides, tendem a apresentar perfil de expressão gênica distinto, enquanto fibrossarcomas, sarcomas pleomórficos e alguns leiomiossarcomas não apresentam características gênicas capazes de diferenciá-los dos demais. (21, 22, 23, 25). Recentemente, Baird et al (35) publicaram a maior série de tumores mesenquimais estudadas através de cDNA microarrays. Os 181 casos estudados, só vieram a confirmar os achados já publicados anteriormente onde alguns sarcomas tem perfis de expressão gênica estabelecidos e outros não. É interessante observar, que aqueles tumores que possuem alterações genéticas específicas já conhecidas como translocações, são os mesmos que possuem perfis de expressão gênica semelhantes e que por isso conseguem se agrupar. Aqueles sarcomas que não possuem alterações específicas, mais uma vez

se mostraram desorganizados do ponto de vista genético. No nosso estudo, o maior grupo avaliado com alteração genética específica foram os sarcomas sinoviais, e também pudemos observar o seu perfil de expressão gênica, distinto o suficiente para separá-los dos demais grupos. (figura 1) Dos genes diferencialmente expressos encontrados, o mais significativo foi o TLE 1. Este mesmo gene já foi reportado por diversos outros estudos de cDNA microarray, realizados por grupos independentes. (36, 37) Ele tem sido considerado um excelente discriminador entre sarcomas sinoviais e outros sarcomas, incluindo aqueles com morfologia semelhante como tumores malignos da bainha do nervo periférico e fibrossarcomas. Proteínas TLE1 são corepressores transcricionais que inibem vias de sinalização como, por exemplo, WNT. Tal fato é corroborado com a morfologia do sarcoma sinovial, a qual não conseguimos relacionar a um tecido diferenciado presente no organismo humano.

Um achado significativo deste estudo foi a respeito das fibromatoses. As fibromatoses músculo aponeuróticas, também chamadas de tumores desmóides, apresentam um comportamento biológico muito peculiar. São extremamente agressivas localmente, capazes de infiltrar tecidos vizinhos, inclusive tecidos ósseos, mas não possuem potencial metastático. Já os fibrossarcomas, também de origem fibroblástica, se comportam como os demais sarcomas. As fibromatoses mostraram ser um grupo bastante coeso e também com perfil de expressão gênica característico, capazes de distinguí-las de outros tumores mesenquimais, tanto malignos como benignos. (figura 1) Este grupo se mostrou tão distinto

que fomos capazes de encontrar 92 trios e 92 pares de genes capazes de separá-las com 100% dos fibrossarcomas e 3 trios capazes de separá-las com 99% de acerto de todos os outros tumores avaliados. (figura 3A e 3B). Os 10 pares e trios com maior p valor podem ser observados nas tabelas 2 e 3. Skubitz et al(38,39) compararam o perfil de expressão gênica de 12 fibromatoses com tecido muscular esquelético normal e encontraram 170 genes capazes de distinguí-las do tecido normal. Dos genes encontrados os mais relevantes foram ADAM12, WISP-1, SOX-11 e FAP alfa. Comparadas a outros tumores mesenquimais, observou a hiperexpressão de colágenos I, V, XI, XII e VI-alfa 2, spondin-1, adlican, fibromodulina, TGF-beta3 e ADAM 12 nas fibromatoses. No nosso estudo os principais genes diferencialmente expressos entre fibromatoses e fibrossarcomas foram SNAP25, NTPD7, MYO5A, TMEM66 e USP1 e entre fibromatoses e demais tumores foram ENTPD7,RNF167,SETD7,FUCA1 e VPS39. Os 30 genes mais diferencialmente expressos assim como seu p valor podem ser vistos na tabela 1. Quando analisamos diversas vias metabólicas , vimos que FGFR1 e PDGFRL, ambos da via do braf, parece discriminar as fibromatoses.

Tais achados podem ser extremamente importantes uma vez que as fibromatoses podem representar um modelo interessante para se estudar o comportamento biológico de diversos tumores, por possuírem características presentes tanto em tumores mesenquimais malignos, como a capacidade de infiltração local e em tumores mesenquimais benignos como a ausência de metástases. A descoberta de genes envolvidos nestes dois

mecanismos pode ser de grande valia para o entendimento do comportamento biológico de diversas neoplasias.

Anexo 2 – Trabalho apresentado no XXVI CBP, Bento Gonçalves-RS.

Tabela 1: 30 genes mais diferencialmente expressos entre Fibromatoses (FM) e Fibrossarcomas (FS)

Genes mais diferencialmente expressos entre FM e FS	*P valor*
SNAP25	-10.024
ENTPD7	-9.730
MYO5A	-9.673
TMEM66	9.581
USP1	8.986
TBX2	8.308
EPB41L2	8.140
C13orf18	-7.917
GREM1	-7.917
AKAP7	7.480
KIAA0367	-7.400
EDIL3	-7.239
GBAS	6.934
CAPN10	6.771
C9orf19	-6.762
PDE4A	-6.723
EXOSC1	-6.464
SQRDL	-6.453
CASD1	6.439
ROBO1	-6.402
MAPK6	-6.261
SGPL1	-6.225
GORASP2	-6.207
ZMYND12	-6.189
UBXD2	6.178
PGM1	-6.165
UNC84A	6.100
IL1R2	-6.087
SDC1	-6.052
SEMA4A	-5.926

Nota: (-) significa genes hipoexpressos e os demais hiperexpressos. FM em relação a FS.

Anexo 2 – Trabalho apresentado no XXVI CBP, Bento Gonçalves-RS.

Tabela 2: 10 Principais trios de genes capazes de separar FM de FS

Trio de genes			*Porcentagem de acerto*	*SVD*
IER3IP1	CDC42EP4	PLAU	100	26.98
MYO5A	FBXO28	PLAU	100	25.413
CDC42EP4	GNG2	PLAU	100	23.434
C1orf165	FBXO28	PLAU	100	22.536
AKAP7	FBXO28	PLAU	100	22.511
NEK7	ZNF655	FBXO28	100	22.027
GREM1	GNG2	FBXO28	100	19.836
GORASP2	FBXO28	PLAU	100	19.723
PLAU	DNAH1	FBXO28	100	19.468
EDIL3	UBE2R2	FBXO28	100	19.427

Tabela 3: 10 principais pares de genes capazes de separar FM de FS

Pares de genes		*Porcentagem de acerto*	*SVD*
CDC42EP4	GNG2	100	22.331
AKAP7	FBXO28	100	21.451
GREM1	GNG2	100	17.846
SDC1	UBE2R2	100	16.564
IHPK2	PLAU	100	16.056
UNC84A	FBXO28	100	15.836
PDE4A	PLAU	100	15.562
GPR180	FBXO28	100	15.216
SLC27A3	PLAU	100	14.875
MYO5A	FBXO28	100	14.608

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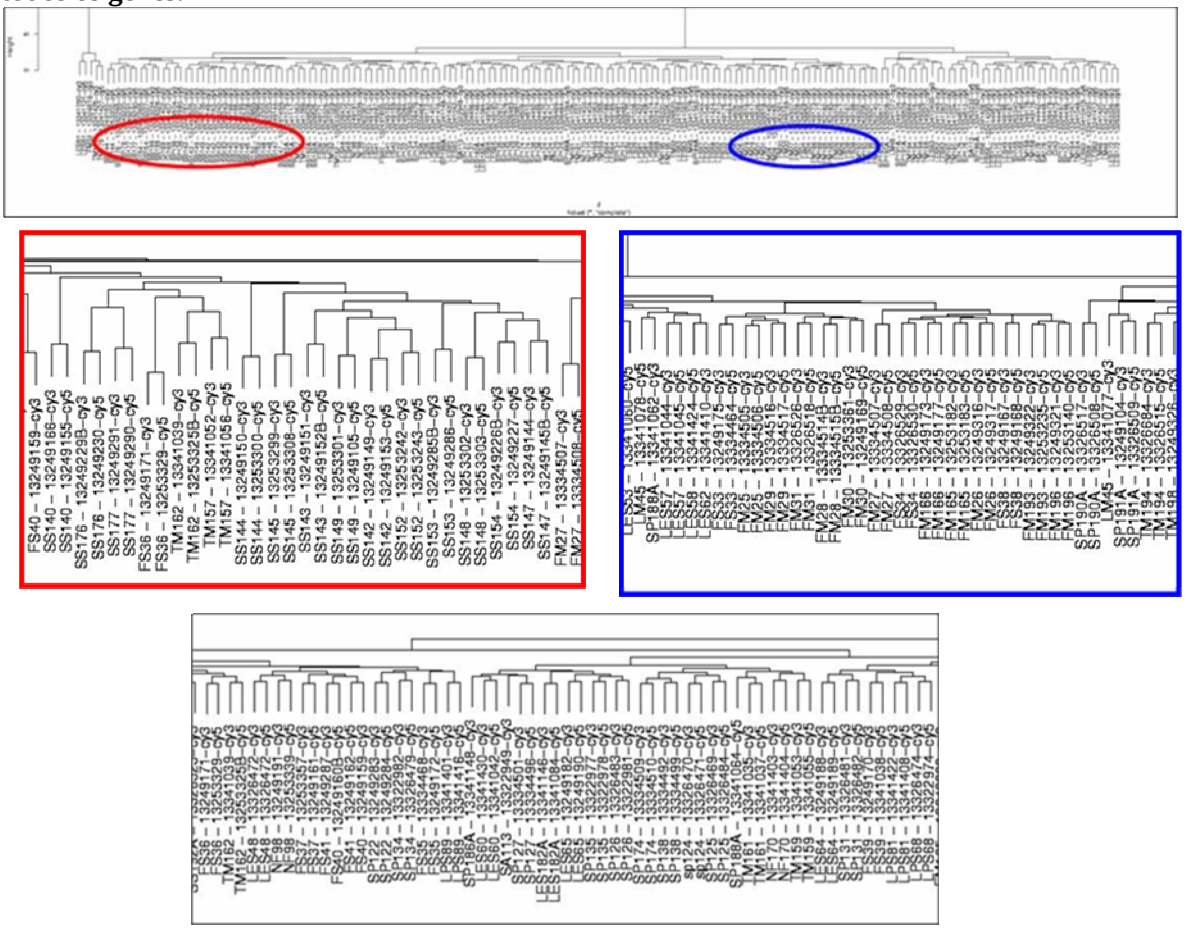
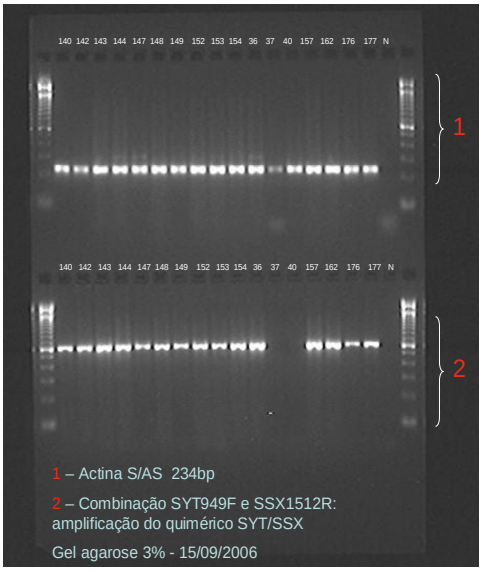
Figura 1: Dendograma mostrando a distribuição das amostras, quando analisados todos os genes.



Anexo 2 – Trabalho apresentado no XXVI CBP, Bento Gonçalves-RS.

Figura 2: Detecção da translocação t X;18 (SSX;SYT) através de RT PCR em Sarcomas sinoviais (SS), e casos classificados morfologicamente como TMBNPs (TM) e Fibrossarcomas (FS).



1. SS140
2. SS142
3. SS143
4. SS144
5. SS147
6. SS148
7. SS149
8. SS152
9. SS153
10. SS154
11. FS36
12. FS37
13. FS40
14. TM157
15. TM162
16. SS176
17. SS177
18. Cont. Negativo

Figura 3: Gráfico esquemático mostrando a distinção entre A - Fibromatoses (verde) e Fibrossarcomas (vermelho), B - Fibromatoses (verde) e demais tumores (vermelho) e C - Sarcomas Sinoviais (verde) e demais tumores (vermelho) utilizando-se o perfil de expressão dos principais trios discriminatórios.

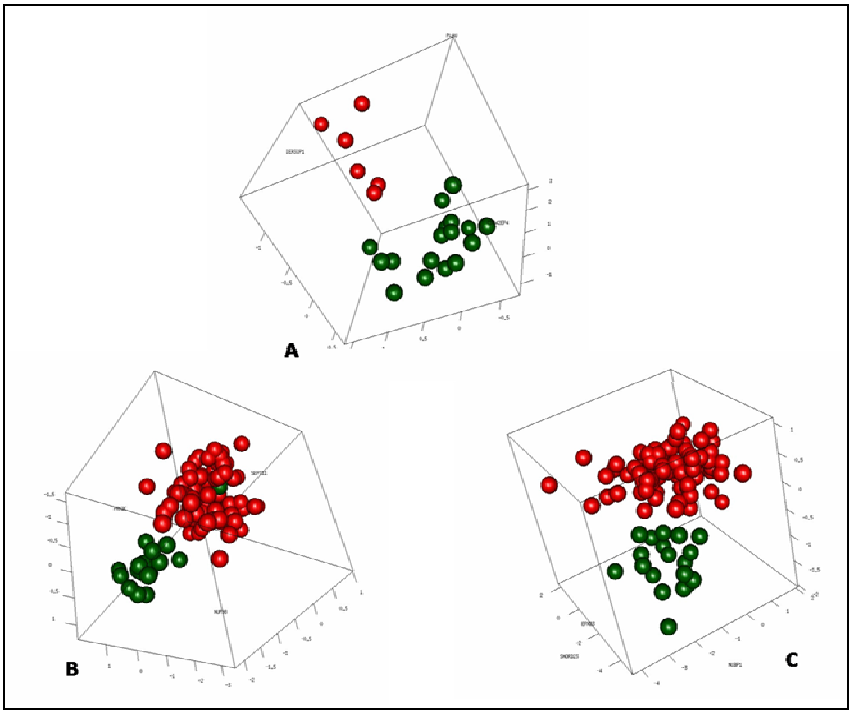
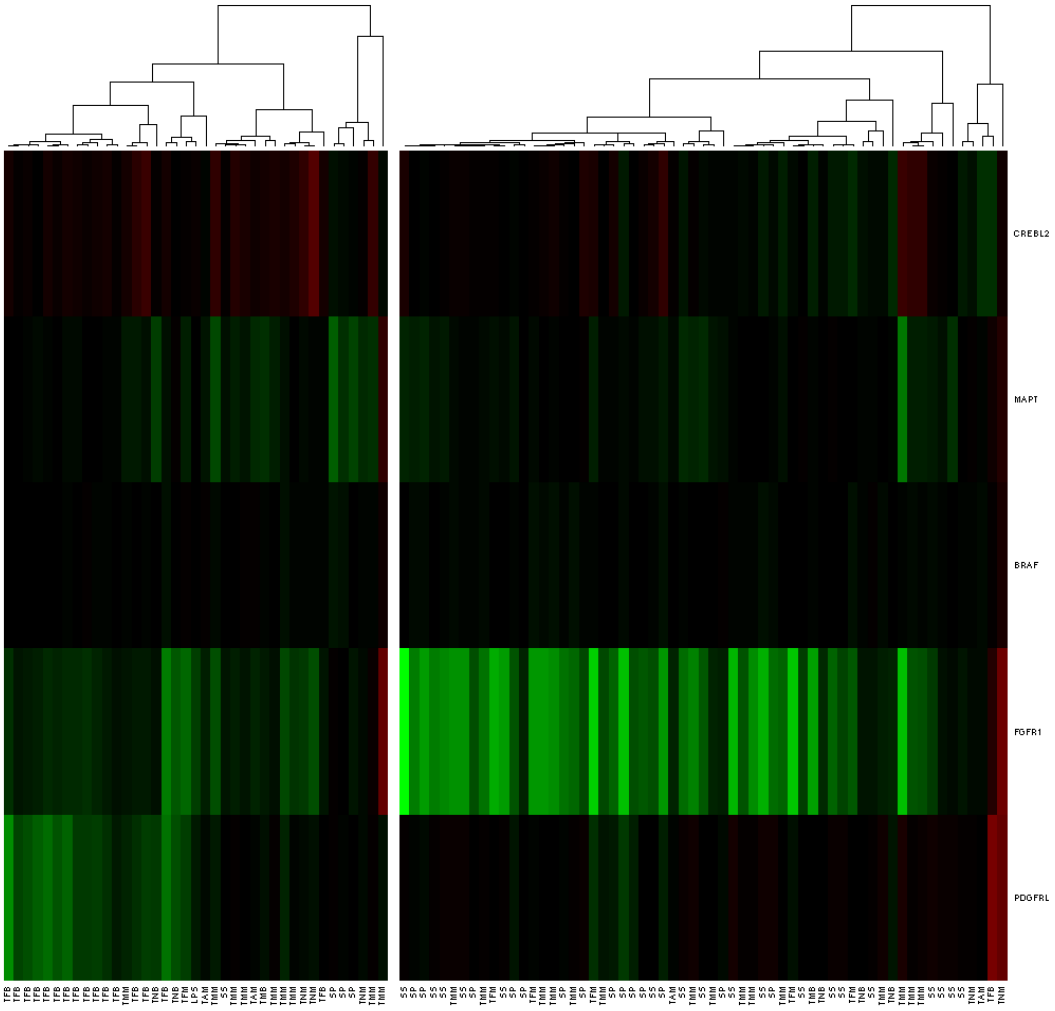


Figura 4: Agrupamento não supervisionado das amostras de sarcoma com base no perfil da expressão de genes pertencentes à via metabólica BRAF. As amostas foram agrupadas por SOM (Self Organizing Map) em dois grupos, com base no perfil de expressão de genes pertecentes à via metabólica B-RAF. Em cada grupo, as amostras foram ordenadas hierarquicamente por distância de correlação. A caracterização das amostras está descrita em material e métodos.



Anexo 2 – Trabalho apresentado no XXVI CBP, Bento Gonçalves-RS.

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Anexo 2 – Trabalho apresentado no XXVI CBP, Bento Gonçalves-RS.

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Anexo 3

Resumo apresentado no AACR 100th Annual Meeting, em abril de 2009 em Denver, CO, USA.

AACR 100th Annual Meeting 2009: Poster Session Abstract #5953

support@abstractsonline.com
Para katia.candido@hcancer.org.br
Data ter 3/2/2009 20:03

February 3, 2009

Re: AACR 100th Annual Meeting 2009 in Denver, CO
Temporary Abstract Number: 5953
Title: **Pairs of genes that separate synovial sarcomas from other histological sarcoma types**

Dear Dr. Carvalho:

Your above-referenced abstract has been scheduled for presentation in a Poster Session at the 2009 AACR Annual Meeting in Denver, CO and will be published in the 2009 Proceedings of the American Association for Cancer Research. Presentation information pertaining to your abstract is below:

Session ID: Cellular and Molecular Biology 30
Session Date and Time: Monday, April 20, 2009, 1:00 PM
Location: Hall B-F, Poster Section 22
Permanent Abstract Number: 2554

Please refer to the printed Final Program (distributed onsite) or the online Annual Meeting Itinerary Planner [available in mid-March through the AACR Website at **http://www.aacr.org**] for the exact location of your presentation.

Instructions for Presenters in Poster Sessions can be found on the 2009 AACR Annual Meeting home page:
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Poster Session presenters at the AACR Annual Meeting must register for the full meeting at the rate appropriate to their membership status and obtain their own hotel accommodations. Registration and housing information are included below:

Advance Registration Deadline: March 10, 2009
Online Registration
http://www.aacr.org/home/scientists/meetings--workshops/aacr-100th-annual-meeting-2009/registration.aspx

Housing Deadline: March 11, 2009
Online Housing System
http://www.aacr.org/home/scientists/meetings--workshops/aacr-100th-annual-meeting-2009/accommodations.aspx

Online Travel Information and Reservation System
http://www.aacr.org/home/scientists/meetings--workshops/aacr-100th-annual-meeting-2009/travel.aspx

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Thank you for your participation in the 2009 AACR Annual Meeting.

Sincerely,

Michael A. Caligiuri, M.D.
Program Committee Chairperson

PLEASE NOTE: This document is your official notice of acceptance. No separate letter of acceptance will be mailed.

Pairs of genes that separate synovial sarcomas from others histological sarcomas types.

Carvalho KC, Cunha IW, Simões AC, Neves EJ, Reis LF & Soares FA

Introduction: Soft tissue tumors (STT) comprise both malignant and benign tumors arising within the extra skeletal connective tissue. Among STT, synovial sarcoma represents a diagnostic challenging, since it shows histological features similar to others spindle cell tumors such as MPNST, fibrosarcoma, PNETs and solitary fibrous tumor. It is very important to correctly classify those tumors since the treatment can differ among STT types. **Objective:** The aim of this study was to determine gene expression profile in STT using cDNA microarray, and define signatures capable of discriminating between synovial sarcomas from other STT. **Material and Methods:** Using a cDNA platform representing 4608 genes, we looked for signatures that discriminate synovial sarcomas (14 samples) from other histological types of STT (51 samples including: fibromatosis, fibrosarcoma, leyomiosarcoma, liposarcoma, MPNST, GIST, and others). Validation of differential expression was done by immunohistochemistry for protein and quantitative RT-PCR (QRT-PCR, with TaqMan detection system) for *FZD1 (Frizzled1), TLE-1* (Transducin – like enhancer of split 1)*, Enfhrin B3* and *PLOD2 (*Procollagen-lysine, 2 – oxoglutarate 5-dioxygenase 2*)* mRNAs. We analyzed 65 samples by QRT-PCR, and 102 samples fixed in formalin and embedded in paraffin by immunohistochemistry. Mann-Whitney test was used for statistical analysis. **Results:** cDNA microarray results pointed a set of genes that discriminate synovial sarcomas from the others histological types of STT analyzed. Among differential expressed genes identified in our analysis, the genes pairs *FZD1/PLOD2, TLE-1/PLOD2* and *Enfhrin B3/PLOD2* precisely discriminates synovial sarcomas from the remaining samples (figure 4). The QRT-PCR confirmed that synovial sarcomas have higher *FDZ1, TLE-1* and *Enfhrin B3* transcript levels than all others STT histological types (figures 1, 2and 3). On the other side, *PLOD2* showed lower expression levels in synovial group. Statistical analysis showed significant differences for *FZD1* ($p<0,0048$), *TLE-1* ($p<0,0001$), *ENFHRIN B3*

(p<0,0001) and *PLOD2* (p<0,0067) genes. Immunohistochemical analysis shown significant differences among histological tumors subtypes stained to Tle-1 and Enfrin B3, but not to Frizzled 1 protein (figures 1,2 and 3). **Conclusions:** Our results indicated that *FZD1/PLOD2, TLE-1/PLOD2* and *Enfhrin B3/PLOD2* genes pairs represents potential diagnostic molecular markers for synovial sarcoma. Together or individually, all these genes could precisely discriminate synovial sarcoma from others mesenchymal tumors.

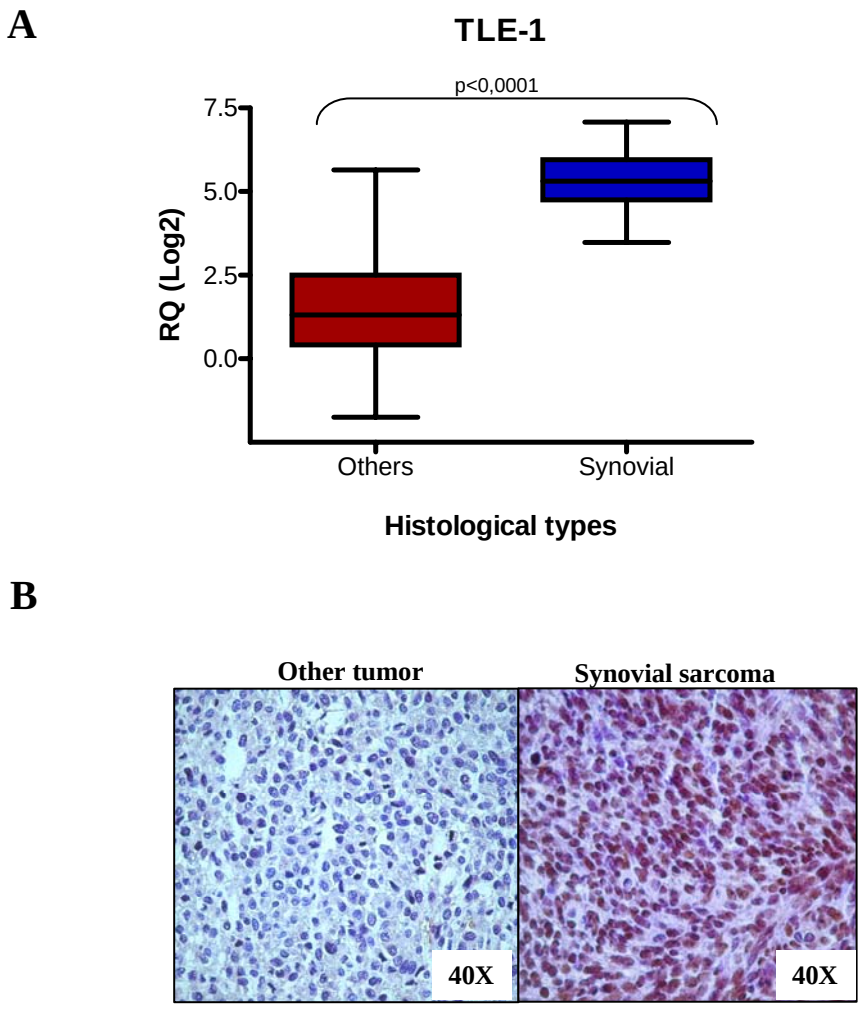
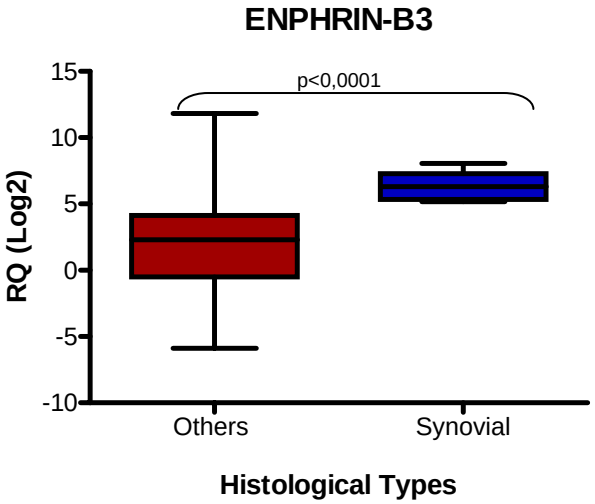


Figure 1: TLE-1 (Transducin – like enhancer of split 1) gene and protein expression analysis. Panel **A:** Real Time PCR results of TLE-1 gene expression using TaqMan probes. Synovial sarcoma (14 samples) showed higher levels of TLE-1 expression than other mesenchymal tumors (51 samples). Expression values were represented in Log2. **B: TLE-1** immunohistochemical reactions showing a negative sample (Pleomorphic sarcoma) and positive sample (synovial sarcoma).

A



B

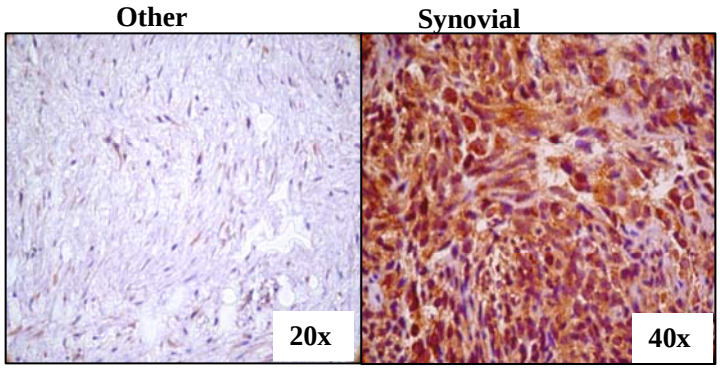


Figure 2: ENPHRIN B3 gene and protein expression analysis. Panel **A:** Real Time PCR results of ENPHRIN B3 gene expression using TaqMan probes. Synovial sarcoma (14 samples) showed higher levels of gene expression than other mesenchymal tumors (51 samples). Expression values were represented in Log2. **B:** immunohistochemical representative reactions showing enfrinB3 protein in negative sample (Desmoid type fibromatosis) and positive sample (synovial sarcoma).

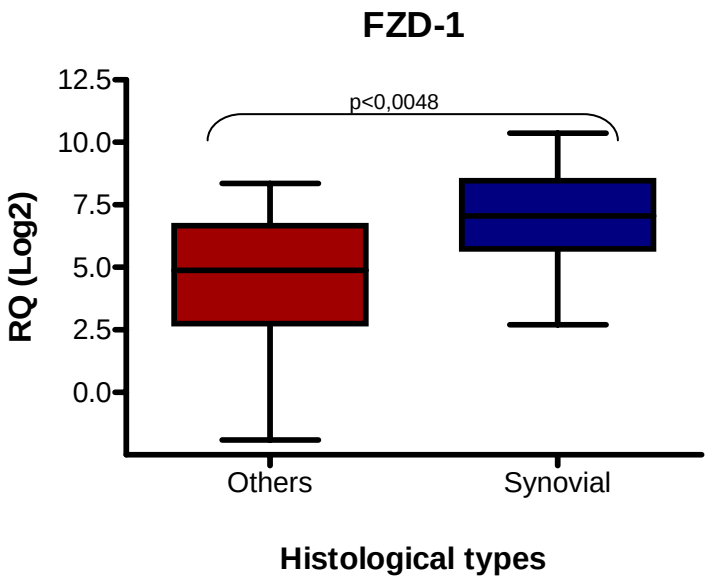


Figure 3: *FZD1* (*Frizzled1*) gene expression analysis by Real Time PCR using TaqMan probes. Synovial sarcoma (14 samples) showed higher levels of *FZD1* gene expression than other mesenchymal tumors (51 samples). Expression values were represented in Log2.

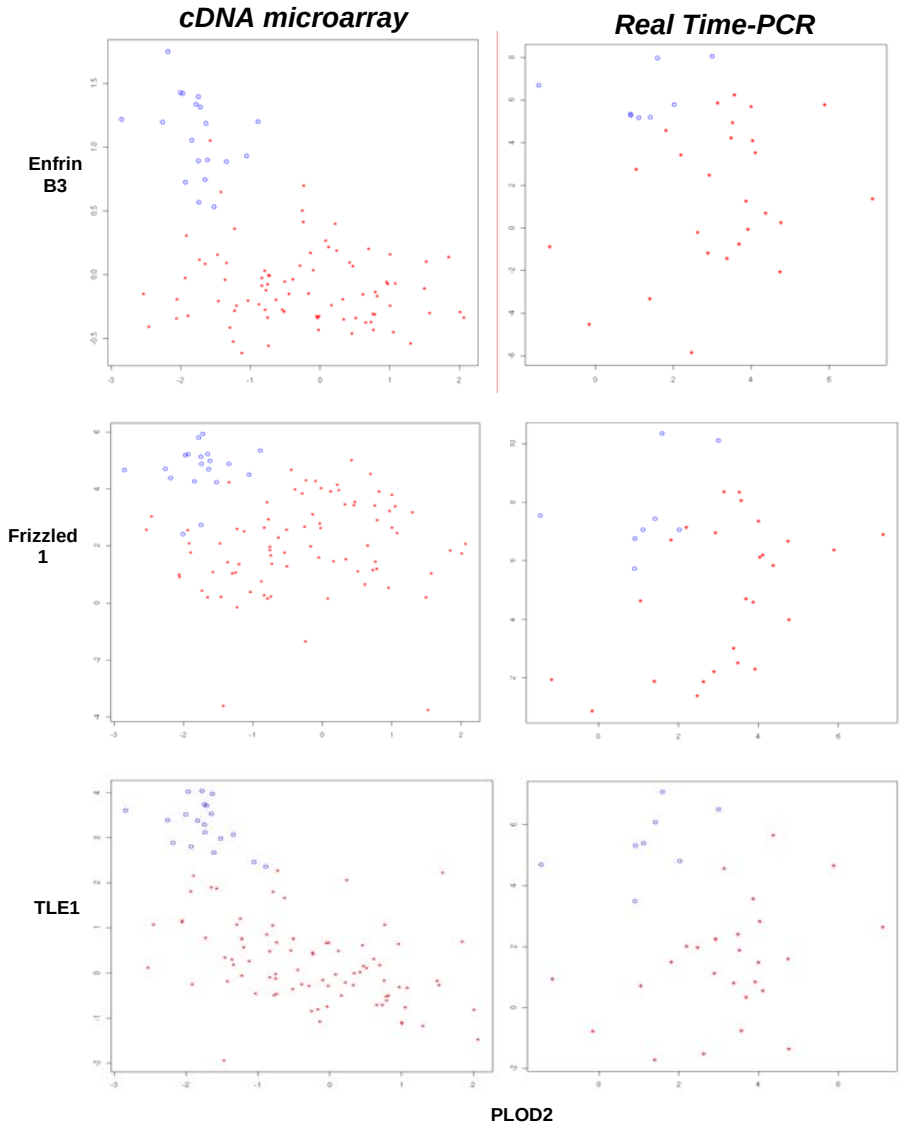


Figure 4: Correlations of transcript expression levels in cDNA microarray and Real Time PCR assays. TLE-1, FZD1 and ENPHRIN B3 were analized in relation of *PLOD2* (Procollagen-lysine, 2 – oxoglutarate 5-dioxygenase 2) gene expression. All analysis showed PLOD2 with inverse expression profile than observed in TLE-1, ENPHRIN B3 and FZD1.

Anexo 4

Artigo publicado na revista “Modern Pathology” em setembro de 2009

Prêmio Erviegas de melhor trabalho apresentado por residente no XXVI Congresso Brasileiro de Patologia, Bento Gonçalves RS, Novembro de 2007

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DIPLOMA

1º Lugar

É concedido o prêmio Erviegas à Dra. **Gisele Caravina Santos**, pela apresentação do trabalho **EXPRESSÃO DE GFAP EM TUMORES MESENQUIMAIS. DESTAQUE PARA A POSITIVIDADE EM TUMORES ÓSSEOS E COM DIFERENCIAÇÃO CARTILAGINOSA: ESTUDO DE EXPRESSÃO GÊNICA E PROTÉICA.**

Bento Gonçalves, 17 de Novembro de 2007.

Carlos Roberto Medeiros
Diretor Presidente

Dr. Luiz Antônio Rodrigues de Freitas
Presidente SBP

Dr. Carlos Thadeu Schmidt Cerski
Presidente do XXVI Congresso

Modern Pathology (2009) 22, 1321–1327

Glial fibrillary acidic protein in tumor types with cartilaginous differentiation

Giscle Caravina Santos[1], Kátia Candido Carvalho[2], Roberto Falzoni[2,3], Ana Carolina Q Simoes[4], Rafael Malagoli Rocha[2], Ademar Lopes[5], Jose Vassallo[2,6], Luiz Fernando Lima Reis[7], Fernando Augusto Soares[2,8] and Isabela Werneck da Cunha[2]

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Glial fibrillary acidic protein (GFAP) is a member of the intermediary filament protein family. It is an important component of astrocytes and a known diagnostic marker of glial differentiation. GFAP is expressed in other neural tumors and pleomorphic adenoma and, less frequently, in cartilage tumors, chordomas, and soft tissue myoepitheliomas. The aim of this study was to evaluate the role of GFAP and its reliability in nonglial tumors as an immunohistochemical marker. We evaluated GFAP gene and protein expression using Q-PCR and immunohistochemistry, respectively, in 81 and 387 cases of soft tissue, bone tumors, and salivary pleomorphic adenomas. Immunohistochemistry staining for GFAP was observed in all osteosarcomas (8 cases), all pleomorphic adenomas (7 cases), in 5 of 6 soft tissue myoepitheliomas, and in 21 of 76 chondrosarcomas. By Q-PCR, GFAP was highly expressed in pleomorphic adenomas and, to a lesser extent, chondrosarcomas, soft tissue myoepitheliomas, and chondroblastic osteosarcomas. The results that we obtained by immunohistochemistry and Q-PCR were well correlated. GFAP is a potential marker for tumors with cartilaginous differentiation, supported by evidence that GFAP is expressed in certain cases of myoepithelial tumors by immunohistochemistry, including soft tissue myoepitheliomas, which are related to cartilaginous differentiation. These findings contribute significantly to the diagnosis of soft tissue myoepitheliomas with cartilaginous differentiation and chondroblastic osteosarcoma in mesenchymal tumors.
Modern Pathology (2009) **22,** 1321–1327; doi:10.1038/modpathol.2009.99; published online 7 August 2009

Keywords: mesenchymal tumors; cartilaginous differentiation; GFAP; immunohistochemistry; Q-PCR

Intermediate filaments are proteins that are associated with plasma membranes and have structural function, reinforcing, and organizing cells in tissues. Their most important function is to provide mechanical support for the plasma membrane, which is responsible for contact with cells or with the extracellular matrix.

Intermediate filaments are grouped into six major classes: type I (acidic keratins), type II (basic keratins), type III (mesenchymal tissues intermediate filaments), type IV (neurofilaments), nonstandard type IV, and type V (laminins). The type III intermediate filament group comprises vimentin, desmin, peripherin, and glial fibrillary acid protein and is found in many tissues. One of the notable molecular aspects of the type III intermediate filament is its capacity to form hetero- and homopolymeric intermediate filaments.[1]

Glial fibrillary acidic protein (GFAP) is a type III intermediate filament. It is a 51-kD protein and has the smallest HEAD domain among the type III intermediate filament proteins. As do other type III intermediate filaments, the assembly of GFAP is regulated by phosphorylation–dephosphorylation of the HEAD domain, wherein its charge is altered.

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This assembly contributes to extensive remodeling of glial frameworks during mitosis.

Another type III intermediate filament protein, vimentin, colocalizes with GFAP in immature, reactive, and radial glia, suggesting that vimentin regulates glial architecture.[1]

GFAP is the major component of several cell types of neuroglia, such as astrocytes, ependymal cells, and Muller cells of the retina. In the central nervous system, it is expressed in ependymal cells and choroid plexus epithelium but is absent from mature oligodendrogliocytes.[2] Among nonglial tissues, GFAP is expressed in Schwann cells, Küpffer cells of the liver, and interstitial cells of the pituitary gland and paraganglions.[2] In addition, GFAP is expressed in osteocytes of mature bones and chondrocytes from epiglottal, bronchial, and tracheal cartilage.[2–6]

GFAP is the most specific marker for cells of astrocytic origin under normal and pathological conditions. By immunohistochemistry, however, GFAP has been shown to be expressed in mesenchymal tumors, such as soft tissue myoepitheliomas, peripheral nerve sheath tumors, and chordomas,[3,7–12] and also in mixed tumors, such as salivary gland pleomorphic adenomas.[13,14] These findings can create problems for the diagnosis of tumors that have similar morphologies and are positive for GFAP, such as myoepithelioma and chondroblastic osteosarcoma of the head and neck. However, GFAP expression can be useful in the differential diagnosis of mesenchymal tumors, especially for small biopsies.

In this study, we evaluated GFAP expression using real-time PCR and immunohistochemistry in mesenchymal tumors and salivary pleomorphic adenomas.

Materials and methods

Samples

Eighty-one tumors from the tissue biorepository of Hospital A. C. Camargo, Sao Paulo, Brazil, were used for Q-PCR experiments. A detailed description of the samples is presented in Table 1.

All samples were collected during surgical procedures from 1997–2007. Tissue samples that were obtained from surgeries were snap-frozen in liquid nitrogen, and biopsy samples were collected in RNAlater® (Ambion®, Austin, TX, USA). All samples were stored at −140°C until further processing. Histopathological diagnoses were reviewed by two pathologists (IWC, RF) before RNA extraction. Frozen samples were hand dissected to remove necrotic tissue and to enrich the tumor. Total RNA was extracted using Trizol® (Life Technologies, Inc., Grand Island, NY, USA).

For the immunohistochemical study, 387 formalin-fixed, paraffin-embedded tumors were obtained from the anatomical pathology files of Hospital A. C. Camargo. All cases were reviewed by two pathologists (IWC, RF), and immunohistochemistry was performed using a panel of antibodies for proper tumor classification.

Table 1 Number of samples of each histological type submitted to immunohistochemistry and Q-PCR for GFAP protein and gene expression evaluation

Histological tumor type	*Immunohistochemistry*	*Q-PCR*
Soft tissue myoepitelioma	06	03
Chondrosarcoma	76	06
Osteosarcoma	08	12
Fibromatosis	19	09
Fibrosarcoma	21	03
Leiomyoma	02	02
Leiomyosarcoma	62	10
Alveolar soft part sarcoma	09	02
Pleomorphic sarcoma	50	10
Synovial sarcoma	57	14
Liposarcoma	48	03
Myxofibrosarcoma	12	–
Angiosarcoma	07	–
PNET[a]	03	–
Pleomorphic adenoma	07	07
Total of samples	387	81

PNET, primitive neuroectodermal tumor.

A detailed description of the samples is presented in Table 1. The analyzed samples were spotted onto tissue microarray slides, as described.[15]

Immunohistochemistry

Tissue sections were mounted on silane-coated glass slides (3-aminopropyltriethoxysilane) and dried for 30 min at 37°C. The sections were deparaffinized in xylene and rehydrated through a series of graded alcohols. Endogenous peroxidase activity was blocked by incubating the sections in a methanol bath that contained 3% hydrogen peroxide for 20 min, which was followed by washes in distilled water. All sections were initially submitted to heat-induced epitope retrieval using citrate buffer (pH 6.0).

The primary GFAP antibody (polyclonal, DAKO®, CA, USA, working dilution 1:10 000) was added and incubated for 90 min at room temperature. Preliminary testing was performed in our laboratory to determine the optimal concentration for the primary antibody and to select negative and positive controls using the dilution data that were supplied by the manufacturer. After washing the primary antibody away with phosphate-buffered saline, we add the polymer advance (DAKO) for 20 min.

Freshly prepared diaminobenzidine solution (one drop of 3,30-diaminobenzidine tetrahydrochloride per 1 ml of substrate, DAKO®) was added to each section for 2 min. Diaminobenzidine solution was removed by rinsing with distilled water. The slides were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted using Entelan.[16]

Evaluation of Immunohistochemistry

Staining was evaluated independently by two observers (GCS and IWC). The intensity of staining reactions was divided into three groups with regard to cytoplasmic staining: 0, negative; 1, weak positivity; 2, strong positivity.

Q-Pcr

Aliquots of 2 μg of total RNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA), following the manufacturer's instructions. Primer pairs for real-time PCR (forward CTGGAGGTTGAGAGGGACAA and reverse CTCCAGCGACTCAATCTTCC) were selected with Primer 3.0 (http://frodo.wi.mit.edu). Reactions were performed with 10 ng cDNA, using the SYBR Green Master Mix system according to the manufacturer's instructions.

Reactions were run in duplicate on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems) and analyzed using Sequence Detection Software (version 2.3). The Ct values were transformed to quantities using the comparative Ct method,[17] and normalization factors for each sample were calculated using Genorm software, based on the expression levels of GAPDH, β-actin, and HPRT genes, as described by Vandesompele *et al*.[18] As a reference, we used a pool of RNA that was obtained from 15 distinct human cell lines, and relative expression values were expressed on a logarithmic scale (log 2). One astrocytoma sample was used as a reference for positive GFAP expression in our analysis.

Statistical Analysis

SPSS, version 12.0 was used for statistical analysis; *t* test was used to compare two groups of numerical variables, and ANOVA was used for more than two groups. The Spearman coefficient was used to evaluate the correlation between groups; χ^2 was used to evaluate the difference in frequencies between groups.

Results

Immunohistochemistry

The results of GFAP immunostaining in different histological tumor types are compiled in Table 2; examples are shown in Figure 1. GFAP expression was observed in 100 (26%) cases. Most of the positive cases showed weak positive immunostaining, and 13 (3%) cases expressed GFAP robustly. Strong positive staining was observed in pleomorphic adenomas (5/7), soft tissue myoepitheliomas (2/6), chondrosarcomas (4/76), and chondroblastic osteosarcomas (2/3) (Figure 1).

For statistical analysis of the immunohistochemistry staining, we separated the tumor types in two groups with regard for their potential for chondroblastic differentiation.

GFAP expression in the tumors with potential cartilaginous differentiation (pleomorphic adenomas, chondroblastic osteosarcomas, soft tissue myoepitheliomas, and chondrosarcomas) was significantly higher compared with the group without it ($P<0.001$). Additionally, there were no other mesenchymal tumors that expressed GFAP strongly.

Table 2 Expression of GFAP in mesenchymal tumors and salivary pleomorphic adenomas visually evaluated by immunohistochemistry

Tumor type	*Negative*	*Weak*	*Strong*	*Total*
Tumor with potential cartilaginous differentiation				
Chondroblastic osteosarcoma	0 (0%)	1 (33%)	2 (67%)	3
Chondrosarcoma	55 (72%)	17 (22%)	4 (6%)	76
Myoepithelioma	1 (17%)	3 (50%)	2 (33%)	6
Pleomorphic adenomas	0 (0%)	2 (29%)	5 (71%)	7
Noncartilaginous differentiated tumors				
Osteoblastic osteosarcoma	0 (0%)	5 (100%)	0 (0%)	5
Angiosarcoma	3 (43%)	4 (47%)	0 (0%)	7
Fibromatosis	14 (74%)	5 (26%)	0 (0%)	19
Fibrosarcoma	14 (67%)	7 (33%)	0 (0%)	21
Pleomorphic Sarcoma	39 (78%)	11 (22%)	0 (0%)	50
Liposarcoma	38 (79%)	10 (21%)	0 (0%)	48
Leiomyoma	2 (100%)	0 (0%)	0 (0%)	2
Leiomyosarcoma	48 (77%)	14 (23%)	0 (0%)	62
Synovial sarcoma	50 (88%)	7 (12%)	0 (0%)	57
Myxofibrosarcoma	11 (92%)	1 (8%)	0 (0%)	12
ASP sarcoma	9 (100%)	0 (0%)	0 (0%)	9
PNET	3 (100%)	0 (0%)	0 (0%)	3
Total	287 (74%)	87 (23%)	13 (3%)	387

ASP, alveolar soft part sarcoma; PNET, primitive neuroectodermal tumor.

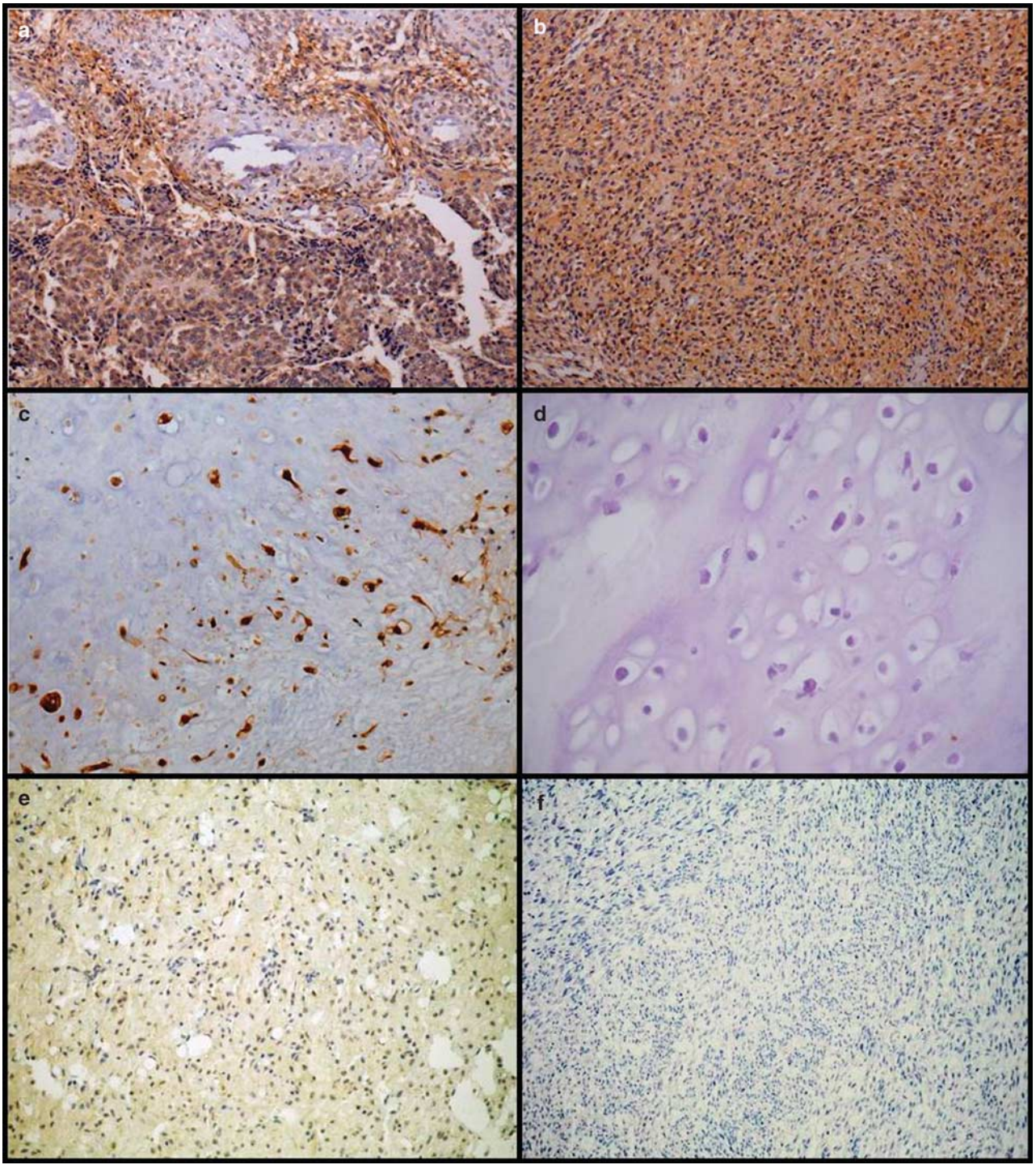

Figure 1 Mesenchymal tumors: immunostaining for GFAP. (**a**) Chondroblastic osteosarcoma (strong positivity, ×100). (**b**) Soft tissue myoepitelioma (strong positivity, ×100). (**c**) Chondrosarcoma (strong positivity, ×200). (**d**) Chondrosarcoma (negative, ×400). (**e**) Liposarcoma (weak positivity, ×400). (**f**) Synovial sarcoma (negative, ×100).

Q-PCR

Overall, tumors that underwent cartilaginous differentiation had higher GFAP expression levels (Figure 2). Considering the histological types of individual tumors, we observed that pleomorphic adenomas ($n=7$) upregulated GFAP, as did soft tissue myoepiteliomas ($n=3$) and chondrosarcomas ($n=4$). There was no significant difference in expression between osteoblastic osteosarcomas ($n=8$) and fibroblastic tumors ($n=7$), pleomorphic sarcomas ($n=6$), leiomyosarcoma ($n=6$), or synovial sarcomas ($n=10$).

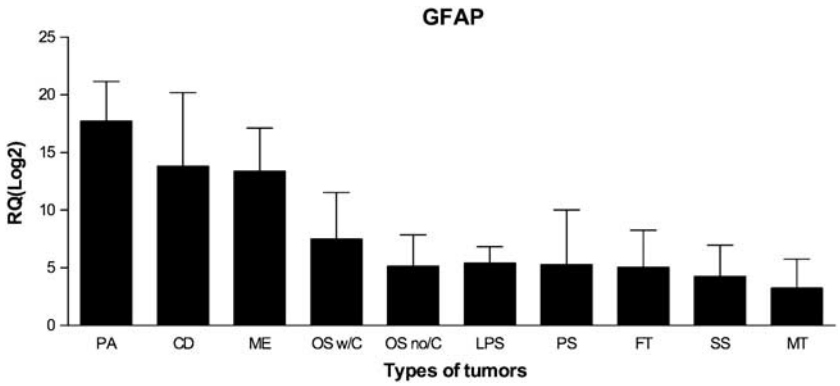

Figure 2 Expression of GFAP evaluated by Q-PCR in pleomorphic adenomas (PA), chondrosarcomas (CD), soft tissue myoepiteliomas (ME), chondroblastic osteosarcomas (OS w/C), osteoblastic osteosarcomas (OS no/C), liposarcomas (LPS), pleomorphic sarcomas (PS), fibroblastic tumors (FT), synovial sarcomas (SS), and muscular tumors (MT). Q-PCR was performed using SYBR green.

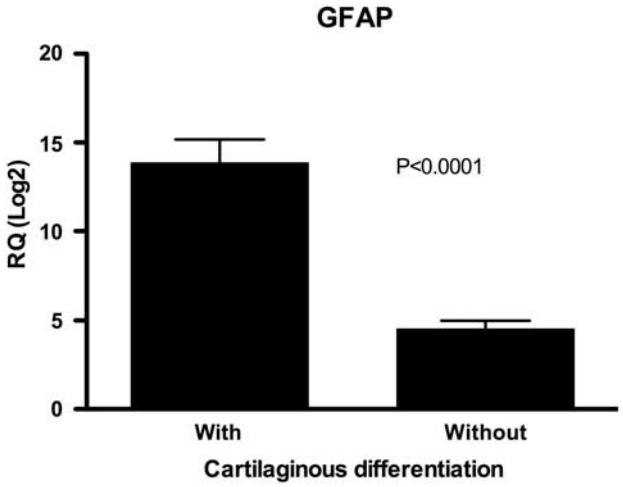

Figure 3 Expression of GFAP, as evaluated by Q-PCR, comparing all tumors that showed cartilaginous differentiation (pleomorphic adenomas, soft tissue myoepiteliomas, chondrosarcomas, and chondroblastic osteosarcomas) with tumors without cartilaginous differentiation (osteoblastic ostesarcomas, synovial sarcomas, liposarcomas, pleomorphic sarcomas, fibroblastic tumors, and muscular tumors).

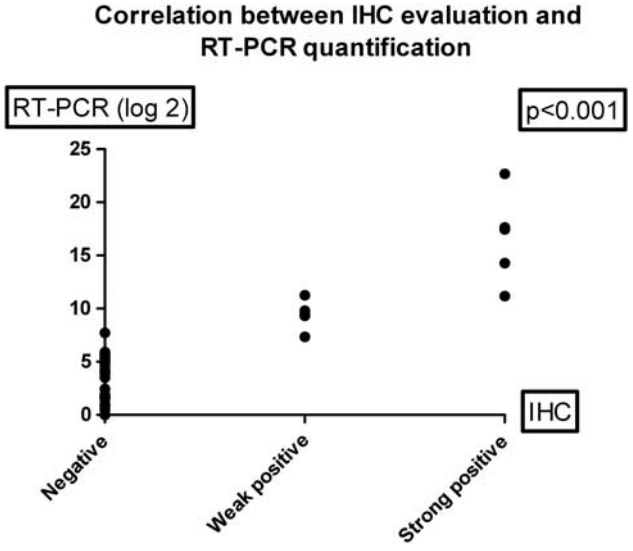

Figure 4 Correlation between immunohistochemistry evaluation and RT–PCR quantification. ($P<0.001$). The Spearman correlation test was used.

We observed a significant difference between tumors that had the potential for cartilaginous differentiation [pleomorphic adenomas ($n=7$), soft tissue myoepiteliomas ($n=3$), chondrosarcomas ($n=4$), and chondroblastic osteosarcomas ($n=4$)] and tumors that did not [osteoblastic ostesarcomas ($n=8$), synovial sarcomas ($n=10$), liposarcomas ($n=3$), pleomorphic sarcomas ($n=6$), fibroblastic tumors ($n=7$), and muscular tumors ($n=9$), including alveolar soft part sarcomas] ($P<0.0001$, Figure 3). GFAP mRNA expression was undetected in 17 sarcoma samples (4 synovial sarcomas, 4 pleomorphic sarcomas, 4 leiomyosarcomas, 3 desmoid-type fibromatosis, and, 2 fibrosarcomas). These cases were excluded from statistical analysis.

For 40 cases, we performed both immunohistochemistry and RT–PCR, and the concordance between immunohistochemistry and RT–PCR expression was highly significant ($P<0.001$), as shown in Figure 4.

Discussion

GFAP expression is the most reliable marker of glial differentiation. We observed an increase in both GFAP mRNA and protein levels in mesenchymal tumors and salivary gland pleomorphic adenomas, most of which presented with cartilaginous differentiation (pleomorphic adenomas, chondrosarcomas, soft tissue myoepitheliomas, and osteosarcomas). Our results are strong evidence that as an intermediate filament, GFAP regulates cartilaginous differentiation.

Expression of GFAP in nonglial cells has been described in mature osteocytes and chondrocytes (13/20 osteocyte samples from the vertebral body, 5/11 chondrocyte samples from the epiglottis, 3/11 chondrocyte samples from rib cartilage, 2/6 samples

from tracheal cartilage, and 4/5 samples from bronchial cartilage),[3] wherein strong cytoplasmatic staining was observed in chondrocytes from bronchial cartilage and tracheal samples stained weakly.[5] These findings suggest that osteocytes and chondrocytes have intermediate filaments in their cytoskeleton that are identical to, are similar to, or associate with GFAP.[3] Chondrocytes in elastic cartilage, stain positive for GFAP more frequently than those from nonelastic cartilage (fibrous or hyaline),[8] as do chondrocytes from the epiglottis. These findings suggest a histogenetic relationship between epiglottal cartilage and the neural crest.[4]

In our samples, 28% (21/76) of chondrosarcomas expressed GFAP at the protein level. Therefore, the majority of chondrosarcomas failed to express GFAP, which can be explained by the observations that immature cartilage increases GFAP expression relative to mature cartilage[5,8] and that the number of GFAP-positive chondrocytes decreases from fetal stages to adulthood.[6]

The etiopathogenesis of chondrosarcomas follows two pathways: originating from a mesenchymal precursor cell or developing from dedifferentiation of mature chondrocytes. Thus, GFAP-positive chondrosarcomas represent unique tumors that originate in immature chondrocytes. Other tumors that experience heterologous cartilage differentiation, such as chondroblastic osteosarcomas, soft tissue myoepitheliomas with chondroblastic differentiation, and pleomorphic adenomas, express GFAP more frequently than chondrossarcomas.

In another study, the authors acquired samples from immature and mature teratomas and classified them into four grades according to their stage of maturity.[8] They showed that GFAP-positive chondrocytes were abundant in immature cartilage and that as the cartilage matured, these chondrocytes decreased in number and distributed peripherally. Furthermore, in immature teratomas, GFAP-positive cartilage neared central nervous tissue.[8] Taking these data into consideration, we speculate that GFAP is expressed in the early stages of differentiation and that GFAP-positive chondrosarcomas originate in immature cartilaginous cells instead of through dedifferentiation of mature chondrocytes.

Another possible explanation for the absence of GFAP in chondrosarcomas concerns the observation that joint cartilage does not express GFAP.[5] These authors reported GFAP immunoexpression in three chondrosarcomas, and in all cases, the expression was restricted to only several cells. They also showed that all joint cartilage cases were negative. We also evaluated all chondrosarcomas by immunohistochemistry with regard to their location (axial or members) and their differentiation (grades 1, 2, or 3), but no differences were found.

Soft tissue myoepitheliomas frequently express GFAP.[7,10–12] In our study, five of six soft tissue myoepitheliomas were positive for GFAP. Cartilaginous differentiation was present in two cases, which were strongly positive, as evaluated by immunohistochemistry. Other authors have described GFAP positivity in 46% of soft tissue myoepitheliomas (40/87) by immunohistochemistry, wherein 24 samples stained robustly.[7] In their study, there were six cartilaginous metaplasia, six tumors that showed osseous differentiation, and four tumors that showed cartilaginous and osseous differentiation. These cases, however, were not evaluated with regard to GFAP expression, and we could not determine whether expression was associated with cartilaginous or osseous differentiation.

GFAP expression also has been reported in an isolated case of gliosarcoma that had characteristics of chondroblastic osteosarcoma, which was confirmed by immunostaining and electronic microscopy.[9]

In our study, all cases of osteosarcomas were positive for GFAP. The strongest expression was documented in tumors with chondroblastic differentiation. In contrast to cartilaginous cells, expression of GFAP is observed in mature osteocytes from vertebral bodies.[3]

Our results, obtained by Q-PCR, which also correlate with the immunohistochemical findings, confirm that GFAP is a bona fide marker for cartilaginous differentiation.

We also measured expression patterns in soft tissue tumors without cartilaginous expression by cDNA microarray in 102 soft tissue tumors, including desmoid-type fibromatosis ($n=19$), fibrosarcomas ($n=6$), malignant peripheral nerve sheath tumors ($n=5$), neurofibromas ($n=3$), schwanomas ($n=2$), leiomyosarcomas ($n=19$), leiomyomas ($n=2$), alveolar soft part sarcomas ($n=2$), gastrointestinal stromal tumors ($n=2$), liposarcomas ($n=6$), synovial sarcomas ($n=19$), and pleomorphic sarcomas ($n=16$). [The accession number for the raw data is GSE14541 (http://www.ncbi.nlm.nih.gov/projects/geo)]. Only one case of leiomyosarcoma and one malignant peripheral nerve sheet tumor upregulated GFAP expression (data not shown). We also compared the pattern of expression between malignant and benign tumors but did not find any difference.

This study suggests that GFAP is related to cartilaginous differentiation. GFAP has been used as a marker for soft tissue myoepithelial tumors. This expression, however, might be because of cartilaginous differentiation, not to myoepithelial cells. Therefore, immunohistochemical expression of GFAP should be performed carefully in the diagnosis of mesenchymal tumors, because not only can soft tissue myoepitheliomas express it but other tumors that are capable of cartilaginous and osseous differentiation can as well.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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