Companion Meeting - USCAP 2017 Annual Meeting
American Association of Ocular Oncologists and Pathologists
Sunday, March 5th, 2017

Diva Regina Salomão, MD

BIOMARKERS IN ORBITAL TUMORS

The National Institutes of Health Biomarkers Definitions Working Group, in 1998, defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological or pathogenic processes, or pharmacologic responses to a therapeutic intervention." More specifically, a biomarker indicates a change in the genetic, epigenetic, proteomics or in the tumor microenvironment that correlates with the progression of disease or that indicates a potential response to a certain treatment. In pathology, in addition to these purposes, biomarkers have also been utilized to assist diagnosis. Herein, we will focus in the biomarkers used to support diagnosis, prognosis, treatment and/or management of a selected group of orbital tumors.

Biomarkers in selected soft tissue tumors of the orbit

About a third of the soft tissue tumors are characterized by chromosomal abnormalities, mostly translocations and amplifications, and these appear to be highly specific. The resulting fusion transcripts not only support the diagnosis, but also provide the basis for the development of new therapeutic strategies aimed at blocking the aberrant activity of the chimeric proteins (Cerrone 2014).

Rhabdomyosarcoma

Rhabdomyosarcomas comprised the single largest category of soft tissue sarcomas in children and adolescents. Orbital rhabdomyosarcomas represent approximately 10% of all rhabdomyosarcoma cases and are the most common malignant orbital tumor in the childhood (Shields 2001). The WHO classification of tumors of soft tissues and bone, divides rhabdomyosarcomas in 4 subtypes (embryonal, alveolar, pleomorphic and spindle cell/sclerosing). The embryonal (ERMS) and alveolar (ARMS) subtypes are the most frequently observed in the orbit.

The importance in differentiating rhabdomyosarcoma from other round cell and spindle neoplasms in the orbit, in addition to recognizing the correct subtype is the implication in treatment and prognosis. Positivity for at least one skeletal-specific marker is required for the diagnosis of rhabdomyosarcoma. MyoD1 and Myogenin are specific and sensitive and considered standard antibodies for the diagnosis, although their expression may be seen only focally in ARMS. Other muscle markers however, such as Desmin and muscle-specific actin (HHF-35) are also seen in other tumors with myogenic phenotype. In addition, aberrant expression of cytokeratin, S100, neurofilament and B-cell proteins (CD20, PAX-5 and immunoglobulins) can be observed in rhabdomyosarcoma.

Analysis of individual genes in sporadic ERMS, have implicated important oncogenic pathways in subsets of ERMS. Deregulation of the RB1 and TP53 pathways is suggested by finding inactivating mutations of *TP53* and *CDKN2B.* The RAS pathway is activated by either mutations on the RAS family

genes or *NF1* deletions. Activation of the hedgehog signaling pathway might occur due to gains in the region containing the *GLI1* gene (12q13) and oncogenic mutations of the *FGFR4* gene occur with or without gene amplification (Paulson 2011). In addition, *PIK3CA* and *CTNNB1* (Beta-catenin) mutations have also been reported (Shukla 2012). All of these findings are potential targets for therapy.

ARMS is consistently associated with recurrent translocations. The t(2;13)(q35;q14) occurs on most cases, while a t(1;13)(p36;q14) occurs in a smaller subset (Barr 2001). These translocations juxtapose *PAX3* or *PAX7* on chromosomes 2 and 1, with *FOXO1* on chromosome 13, to create chimeric genes that encode PAX3- and PAX-7 fusion proteins, which function as potent transcriptional activators with oncogenic effects (Cerrone 2014). Fusion-positive ARMS are notable for frequent genomic amplification; the most common is *PAX7-FOXO1* amplification, but *MYCN* oncogene amplification on 2p24 and amplification of a 12q13-14 region, which includes *CDK4* gene, are also observed. Translocations and amplifications can be tested by a variety of techniques including interphase FISH analysis and PCR.

Patients with ARMS tumors have a poorer outcome than patients with ERMS tumors, highlighting the importance to distinguish these 2 subtypes. Among patients with ARMS, the 4-year failure free survival rates for patients with localized and metastatic ARMS are 65% and 15%, respectively. Traditionally, risk factors that influence outcome of ARMS include primary site, size of primary tumor, extent of local spread, and the presence of nodal and distal metastases (Fletcher 2013). In an analysis of patients from the IRS-IV study, patients with localized PAX3-FKHR and PAX7-FKHR-positive ARMS had comparable outcomes. In contrast, among patients presenting with metastatic disease, those with PAX3-FKHR-positive tumors had a significantly poorer outcome than those with PAX7-FKHR-positive tumors (4-year overall survival rate of 8% compared to 75%, p=0.0015) (Sorensen 2002).

Solitary fibrous tumor-Hemangiopericytoma-Giant cell angiofibroma

Solitary fibrous tumor (SFT) is a neoplasm of variable clinical behavior, composed of spindle to oval cells, in patternless arrangement, rich in collagen and positive for CD34 immunostain (non-specific). The majority of SFTs behavior in a benign fashion, but recurrences and metastases occur in approximately 5-10% of the patients. This latter group usually displays features of malignancy (> 4 mitoses per 10hpf and hypercellularity); however, benign-appearing SFT have been reported to metastasize (Demicco 2012). Patients with orbital SFT may present with painless orbital mass and/or proptosis.

More recently, it has been suggested, based on overlapping morphological features and immunohistochemical findings, that SFT, giant cell angiofibroma, and hemangiopericytoma represent a spectrum of the same lesion and therefore should all be called SFT (Furusato 2010, Fletcher 2013, Tway 2016).

By using whole-exome and transcriptome sequencing, *NAB2-STAT6* gene fusions have been identified in the majority of SFTs (Robinson 2013, Chmieleck 2013). These genes are located closely together on chromosome 12 and are transcribed in opposite directions. The fusion product results from an inversion at the 12q13 locus, which fuses *NAB2* and *STAT6.* The resultant fusion protein, interleukin-4 induced (STAT6), is believed to act as a transcriptional activator, inducing expression of EGR-target

genes, which results in increased proliferation. This same fusion is observed in cases with the morphology of hemangiopericytoma. Nuclear STAT6 overexpression can be demonstrated by immunohistochemistry in nearly all cases of SFT and has proven to be very helpful in differentiating SFT/hemangiopericytoma from other spindle cell tumors, benign (soft tissue perineurioma, desmoid fibromatosis, spindle cell lipoma and cellular angiofibroma), tumors with aggressive behavior (fibromatosis) and spindle cell sarcomas (malignant peripheral nerve sheath tumor,dermatofibrosarcoma protuberans and synovial sarcoma) (Doyle 2013).

Lipomatous tumors

Although adipocytic tumors are common soft tissue neoplasms, these are extremely rare in the orbit. In the AFIP series, it represented less than 1% of all orbital tumors (Font 2006, Schields 2004). A few cases of spindle cell lipoma have been reported (Bartley 1985), as well as a pleomorphic lipoma, an entity that needs to be differentiated from degenerative atypia seen in herniated orbital fat (Daniel 2003). Most lipomas show cytogenetic abnormalities, however, these are not utilized to support diagnosis usually achieved on the basis of morphology alone. The most common abnormalities seen in lipomas are aberrations involving 12q13-15.

The majority of orbital liposarcomas is either well-differentiated or myxoid types and occurs in the retrobulbar space and lateral orbital wall (Cai 2001). Dedifferentiated liposarcomas are rarely seen in the orbit.

The MDM2 (mouse double minute 2 homolog) is a gene located at 12q13-15 that encodes the MDM2 protein, an important negative regulator of the p53 tumor suppressor. This gene is amplified in well-differentiated and also dedifferentiated liposarcomas originating from the former. Overexpression of the MDM2 protein resulting from genomic amplification inactivates TP53. MDM2 targets TP53 degradation toward the proteasome, and inhibits TP53-mediated transactivation (Fletcher 2013). Quantitative detection of MDM2 by FISH (Sirvent 2007), PCR (Hostein 2004), array CGH (Tap 2011), or indirectly by immunohistochemistry (nuclear immunoreactivity) helps to differentiate these malignant tumors from benign adipose tissue tumors. Several other genes located in the 12q14-15 region, including CDK4, HMGA2, YEATS4, CPM and FRS2 are frequently co-amplified with MDM2.

Different from pure well-differentiated liposarcomas, dedifferentiated type is characterized by co-amplifications involving mainly1p32 and 6q23 which include *JUN* and its activating kinase *ASK1* as target genes, suggesting that the c-Jun pathway may be implicated in the progression from well-differentiated to dedifferentiated liposarcomas (Fletcher 2013).

Myxoid liposarcomas are characterized by the recurrent translocation t(12;16)(q13;p11)that results in *FUS-DDIT3* gene fusion, present in > 95% of the cases. In the remaining cases, a variant T(12;22)(q13;12) is present in which *DDT3* (*CHOP*) fuses with *EWSR1* instead (Panagopolous 1996). There are at least 11 different isoforms of the *FUS-DDIT3* fusion transcript and 4 known forms of the *EWSR1-DDIT3* fusion transcript. These different isoforms have not been associated with differences in histological grade or clinical outcome (Bode-Lesniewska 2007).

These alterations in liposarcomas have been used as diagnostic aids in the differential diagnosis with other fat-rich lesions as well as other sarcomas.

Ewing's sarcoma/Peripheral Primitive Neuroectodermal Tumor

Ewing sarcoma is a small, round cell sarcoma showing pathognomonic molecular findings and varying degrees of neuroectodermal differentiation by light or electron microcopy, or immunohistochemistry. Primary orbital ES/pPNET is extremely rare with very few cases reported (Romero R 2011). They are included however in a number of differential diagnoses in the orbit, therefore, included in this discussion.

Cytogenetic studies have identified a common cytogenetic abnormality in the Ewing Sarcoma family, t(11;22)(q24;12) (Delattre 1994). Subsequent studies have found that ES/pPNET are characterized by balanced translocations involving, in almost all cases, the *EWSR1* gene on chromosome 22 and various members of the ETS family of transcription factors, which leads to the formation of novel fusion oncogenes, key on it pathogenesis. EWSR1-ETS fusion proteins activate or repress specific sets of target genes that in the right time and cellular environment give rise to Ewing sarcoma.

Approximately 85% of Ewing sarcomas harbor a somatic reciprocal chromosomal translocation, t(11;22)(q24;q12), that fuses *EWSR1 to FLI1.* The fusion gene encodes and oncoprotein on the domain *FLI1*, resulting in aberrantly active transcription factors capable of DNA binding and malignant transformation (Janknecht 2005). The second most common translocation is t(21;22)(q22;12) leading to the fusion of *EWSR1* to *ERG* at 21q22. Less common are fusions of *EWSR1* to *ETV1* at 7p22 and *ETV4* at 17q12. The ability to detect these fusions by molecular genetic techniques (FISH or RT-PCR) on FFPE tissue, has greatly improved the diagnosis of this family of tumors (Bridge 2006). Also, some studies have found that the type of *EWSR1-FLI1* fusion may be prognostically relevant, as patients with type 1 fusions have been reported to have longer disease-free survival.

Alterations in *TP53* and *p16/p14ARF* are detected in 25% of EFT; these alterations are detected in a subset of patients with an aggressive clinical course, who are also refractory to chemotherapy (Mertens 2009).

<u>Biomarkers in selected lacrimal gland tumors</u>

Lacrimal gland tumors are rare (von Holstein 2013). Much of the knowledge regarding their behavior and molecular pathogenesis is the result of parallels drawn from their equivalent in the salivary glands (White 2012). Ancillary studies such as biomarker immunostains, and more recently, molecular assays for key gene alterations have the potential to supplement traditional parameters used to determine prognosis in these neoplasms.

The most common benign and malignant neoplasms in the lacrimal gland are pleomorphic adenoma (PA) and adenoid cystic carcinoma (ACC), followed by carcinoma ex-pleomorphic adenoma and adenocarcinoma NOS (Shields 2004, Weis 2009).

Pleomorphic adenoma

Pleomorphic adenoma or benign mixed tumor represents approximately 10% of all lacrimal gland tumors. It occurs most frequently in the 5^{th} and 6^{th} decades with no sex predilection. They are grossly well-circumscribed although not encapsulated, and microscopically characterized by a

proliferation of ductal epithelial cells admixed with myoepithelial cells in myxoid, hyaline or chondroid stroma with great variability.

Extensive cytogenetic studies have shown that approximately 70% have abnormalities. Four common subgroups have been identified: group 1 are tumors with rearrangements involving 8q12 (39%); group 2 are tumors with rearrangements of 12q13-15 (8%); group 3 are tumors with sporadic, clonal changes not involving either 8q12 or 12q13-15 (23%); group 4 are tumors with normal karyotype (30%).

The target gene in PAs with 8q12 abnormalities is *PLAG1*. *PLAG1* encodes a zinc finger DNA-binding nuclear oncoprotein that functions as a transcription factor. Translocations centered on *PLAG1* involve a mechanism of promoter swapping with a partner gene, the gene for beta-catenin (*CTNNB1*). The most commonly observed fusions are *CTNNB1-PLAG1* and *LIFR-PLAG1* resulting from t(3;8)(p21;q12) and t(5;8)(p13;q12). A recent study found cryptic gene fusions involving *CTNNB1-PLAG1* and *SII-PLAG1* in karyotypically normal PAs (Barnes 2005).

The target gene in PAs with 12q14-15 rearrangements *HMGA2* (high mobility group protein gene). *HMGA2* encodes an architectural transcription factor that promotes activation of gene expression by modulating the conformation of DNA. Two fusion genes, *HMGA2-NFIB* and *HMGA2-FHIT*, have been identified (Geurts 1998). High level expression of *HMGA2* resulting from gene amplification has been suggested to be of importance in the malignant transformation of Pas (Roijer 2002).

The 5 identified PLAG1- and HMGA2-containing fusion genes are all tumor specific and might be used as diagnostic markers for PA. These can be detected by RT-PCR or interphase FISH.

Adenoid cystic carcinoma

Adenoid cystic carcinoma (ACC) is a biphasic tumor composed of ducts and basal/myoepithelial cells that can be arranged in a tubular, cribriform or solid pattern. The biologic course of this neoplasm is slow but relentless; a 5-year survival is favorable at about 75-80% of patients, but 15-year survival is still poor at about 35-20% (Fordice 1999). Patients with lacrimal gland ACC have a median age of 40 years, and symptoms such as pain and eye displacement are common. Histologically, ACC is graded based on growth pattern with solid growth imparting a poorer prognosis, with a higher likelihood of lymph node metastasis.

Lacrimal gland ACCs, similar to their salivary gland counterpart, display a recurrent chromosomal translocation t(6;9)(q22-23;p23-24). This translocation results on *MYB-NFIB* fusion, with overexpression of *MYB* and its downstream targets. ACC have genomic profiles characterized by losses involving 1p, 6q, 12q, and 17p, and gains involving chromosome 22, 19q, 8q, and 11q (von Holstein 2013). A high number of chromosomal alterations seem to be an adverse prognosticator in salivary gland ACC (Vekony 2007). *MYB-NFIB* has been found to be a useful diagnostic biomarker for ACC and its downstream targets are potential therapeutic targets for these tumors.

Of interest, an aggressive variant of ACC, ACC with high-grade transformation, have p53 alterations in 40% of the cases. Comparative genomic hybridization studies have implicated gains in chromosomal regions 8q24 (C-MYC gene locus), 17q11.2-q12, 17q23 and 15q11-13. This variant has 50% lymph node metastatic rate when compared with the conventional ACC (Seethala 2011, White 2012).

Less common lacrimal gland neoplasms

Carcinoma ex-pleomorphic adenoma of the lacrimal gland is a malignant lacrimal gland tumor component of any type, adenocarcinoma been the most frequent, arising in the background of a benign pleomorphic adenoma. Overexpressions of c-erbB-2 and EGFR have been found in high grade areas of carcinoma ex-pleomorphic adenoma. Alterations of *p53* and p53 protein overexpression suggest that this gene may play a role in the malignant transformation.

Adenocarcinoma NOS is a carcinoma with glandular or ductal differentiation that lacks morphological features to be categorized in a more specific subtype. A recent study, on salivary gland tumors, found mutually exclusive genetic aberrations in 56% of these tumors, including mutations in BRAF (7%), mutations in PIK3CA (19%), HER2 gene amplification (30%). These findings suggest the usage of targeted therapies for patients with this rare malignancy (Nardi 2013).

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