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**CORRESPONDENCE**

# PAX7 expression in sarcomas bearing the *EWSR1-NFATC2* translocation

Gregory W. Charville[1] · Wei-Lien Wang[2] · Davis R. Ingram[2] · Angshumoy Roy[3] · Dafydd Thomas[4] · Rajiv M. Patel [4] · Jason L. Hornick[5] · Matt van de Rijn[1] · Alexander J. Lazar [2]

We thank Dr. Grunewald and colleagues for reading our manuscript [1], and we are happy to respond to their comments. In their letter, Grunewald et al. [2] raise the question of whether *EWSR1-NFATC2*-positive sarcomas really are Ewing sarcomas. They provide information from their interesting publication that appeared in *Oncotarget* to support the idea that *EWSR1-NFATC2*-positive sarcomas are transcriptionally distinct from tumors with *EWSR1-FLI1* translocation. Unfortunately, their manuscript was published after the publication of our paper, and therefore we could not reference their data in our report. They do mention that we did not "take into account more recent reports in the literature that *EWSR1-NFATC2*-positive sarcomas may constitute an own entity." Here, their letter cites a single article summarizing a meeting of the European Interdisciplinary Ewing Sarcoma Research Summit [3], published in 2016. Grunewald et al. feel that we have overlooked this publication, which would have served, in their view, as a guide to the appropriate classification of the *EWSR1-NFATC2* variant tumors. However, the cited publication, albeit a generally helpful and important commentary, which references unpublished gene expression studies

of *EWSR1-NFATC2*-translocated tumors, presents no primary data to distinguish these as a unique entity. Also, this publication does not provide specific recommendations to guide classification of the *EWSR1-NFATC2* variant.

In our decision to analyze *EWSR1-NFATC2* cases as members of the "Ewing family," we followed in the footsteps of several preceding publications that treat *EWSR1-NFATC2* tumors as Ewing sarcoma variants [4–17]. Notably, the College of American Pathologists' [26] *Protocol for the Examination of Specimens from Patients with Soft Tissue Tumors* version 4.0.0.0 (published in June 2017) also lists the *EWSR1-NFATC2* fusion as a "molecular event" in "Ewing sarcoma." For its part, the World Health Organization's [27] *Classification of Tumours of Soft Tissue and Bone* (2013) states in reference to *EWSR1-NFATC2* tumors that "whether these cases should be defined as Ewing sarcoma, or represent separate tumor types is unknown, but currently they are treated in a similar fashion."

We too feel that the question of whether the *EWSR1-NFATC2* tumors should be treated as an entity completely distinct from *EWSR1-ETS* tumors is not yet resolved. However, knowing that the underlying translocation could be relevant to these tumors' eventual classification, we were careful to indicate throughout our manuscript when our analyses involved specific "variants." Regardless of the nomenclature, the *EWSR1-NFATC2* tumors, like the *EWSR1-FLI1* and *EWSR1-ERG* cases, express PAX7, while lesions with a *CIC-DUX4* translocation do not. This was the message of our manuscript. Importantly, a recent RNA-sequencing study of small round-cell sarcomas [18], including gene expression data from 184 tumors, confirmed our observation that *PAX7* is one of the most differentially expressed genes in Ewing sarcoma relative to other small round -cell sarcomas.

We concur with Grunewald et al. that pathologists need to be cognizant of the limitations and specificity of PAX7 expression analyses. In fact, our paper references and

✉ Gregory W. Charville
gwc@stanford.edu

1 Department of Pathology, Stanford University School of Medicine, Stanford, CA, USA

2 Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

3 Departments of Pathology & Immunology and Pediatrics, Texas Children's Hospital, Baylor College of Medicine, Houston, TX, USA

4 Department of Pathology, University of Michigan School of Medicine, Ann Arbor, MI, USA

5 Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA

discusses our prior study in which we found PAX7 to be expressed in both rhabdomyosarcoma and synovial sarcoma [19]. Therefore, like many antibodies employed in surgical pathology, we believe PAX7 would be best used in a panel of markers and in the appropriate clinicopathologic context. Although we did not make any assertions about the sensitivity of PAX7 relative to CD99, we did state that CD99 expression is "highly sensitive" for the identification of Ewing sarcoma and would indeed think it ill-advised to suggest that PAX7, or any marker for that matter, should wholly supplant a "tried and true" diagnostic tool such as CD99. Nevertheless, in our series, all molecularly proven Ewing sarcomas expressed PAX7 and, therefore, we think PAX7 expression may be a useful ancillary diagnostic tool for these tumors.

Grunewald et al. mention the importance of confirming the presence of an *EWSR1* translocation in tumor samples. We also think this is important—in both the "Materials and methods" and the "Results" section of our paper, we describe the molecular characterization of all PAX7-negative cases in our initial cohort study using next-generation sequencing (NGS) of libraries prepared with an anchored multiplex PCR-based methodology. The vast majority of remaining PAX7-positive samples had been confirmed through routine clinical testing by FISH, RT-PCR, or sequencing methodologies, as has been the practice in our institutions for many years.

Grunewald et al. additionally inquire with regard to our characterization of a candidate EWSR1-FLI1-regulated *PAX7* enhancer. We focused on a single candidate regulatory element because it met the following criteria of an EWSR1-FLI1-regulated active enhancer: (1) the site is bound by EWSR1-FLI1 in SKNMC and A673 cell lines, (2) the site coincides with a peak of H3K27 acetylation in SKNMC and A673 cells that is lost with knockdown of the oncogenic fusion protein, (3) the site overlaps with a peak of H3K27 acetylation, which is gained in mesenchymal stem cells upon transgenic expression of the oncogenic fusion protein, and (4) the site coincides with a peak of H3K27 acetylation in three primary Ewing sarcoma specimens [20]. Other candidate EWSR1-FLI1 binding sites proximal to *PAX7*, in our opinion, do not meet these stringent criteria. Still, given the complexity of distal regulatory elements in the human genome, we would not expect, nor did we suggest, that the element highlighted by our analysis represents the sole mechanism of *PAX7* expression in Ewing sarcoma.

Lastly, Grunewald and colleagues point to the polymorphic nature of GGAA-microsatellites as a reason to doubt that such repeats would mediate "uniformly high" *PAX7* expression in Ewing sarcoma. It is well established that EWSR1-FLI1 creates *de novo* enhancers at GGAA microsatellites to increase transcription of target genes [20–23]. The EWSR1-FLI1-bound regulatory element that was highlighted in our study is centered on a microsatellite containing 18 GGAA motifs in the reference genome (hg19). These 18 consecutive motifs meet the established threshold of >10AA repeats required for "strong" reporter gene activity in studies of EWSR1-FLI1 transcriptional regulation and fall just within the range of 18–26 motifs representing the "optimal" length for EWSR1-FLI1-binding [21, 24, 25]. Moreover, analysis of the *NR0B1* promoter GGAA-microsatellite has shown that (1) stability of GGAA microsatellites during oncogenesis limits polymorphism within individual tumors and (2) "selection bias" restricts the case-to-case variability of GGAA-microsatellite length in Ewing sarcoma [24]. These findings predict robust gene expression mediated by a GGAA microsatellite like the one identified in our study.

In this age of precision medicine, topics such as the relationship of *EWSR1-NFATC2* translocated Ewing or "Ewing-like" sarcomas to the canonical *EWSR1-FLI1* translocated Ewing sarcomas are of critical importance, and we thank Grunewald et al. for reminding us of this. At this point, we believe that the jury is still out regarding this distinction and we look forward to the related discussions at the upcoming working group conference of the WHO Classification of Tumours of Soft Tissue and Bone. Establishing thresholds for designating an entity as truly distinct from another is a complex decision involving molecular data, clinical experience and practice, and, to a certain degree, social constructions. Although the designation of *EWSR1-NFATc2* tumors is currently unresolved in our opinion, data such as those presented by Grunewald et al. and our own work, as well as the work of others around the globe, will hopefully bring additional clarity over time.

## Compliance with ethical standards

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