CORRESPONDENCE

Genomic Amplification Is Not a Frequent Event in Uveal Melanomas

To the Editor-in-Chief:

We read with interest the article by Lake et al.¹ and were surprised by the interpretation of their results. Uveal melanomas (UMs) are rare tumors associated with negative prognosis. Risk of recurrence can be predicted by pathological and genetic findings like monosomy of chromosome 3 and gains of 8q.^{2–4} Nonetheless, the identification of new genetic markers is warranted to better stratify patients and unravel the pathogenesis of this tumor.

Lake et al.¹ performed a copy number and genotyping analysis on formalin-fixed, paraffin-embedded (FFPE) samples of 58 cases with the genome-wide SNP arrays version 6.0 (Affymetrix, Santa Clara, CA). They reported an amplification of the *CNKSR3* gene in 11 of 58 cases (19%) and described numerous other genes as amplified. These findings caught our attention because UMs are known for having simple genome profiles defined by very few copy number alterations (involving chromosomes 3, 6, and 8) and recurrent point mutations (*GNA11*, *GNAQ*, *BAP1*, and *SF3B1* genes).^{3,5} Since 2005, several hundred cases of UM have been analyzed^{2,4,6} and actual recurrent amplifications (ie, related to focal amplicons) have not been previously described. To extend this literature series, we reviewed 300 comparative genomic hybridization profiles from our data obtained on NimbleGen 4x72K whole genome arrays (Roche NimbleGen, Reykjavik, Iceland) and did not detect any *CNKSR3* amplification. As no threshold for genomic amplification was defined in the article by Lake et al.,¹ we hypothesized that amplification was defined with a low copy number threshold corresponding in fact to a chromosomal gain. To test this hypothesis, we retrieved the publicly available raw data used for this publication from Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>; accession number GSE37259). We reanalyzed the data using the standard manufacturer pipeline with Genotyping Console and Birdseed version 2 algorithm (Affymetrix) as recommended for SNP6.0 arrays rather than Birdseed version 0 as used by the authors. Some have recommended to modify the final *in silico* data analysis procedure to extract most of the biological information from the signal measured.⁷ Using the above mentioned rigorous procedure,

we could not confirm the findings from Lake et al.¹ The principal issue was the low quality control call rates that impeded our ability to retrieve reliable genotyping and copy number information. Such low quality SNP array profiles are expected when using FFPE samples because of the degradation of genomic DNA. Therefore, we think that extreme caution should be taken when interpreting such profiles, especially regarding the presence of recurrent amplifications or the absence of isodisomy of chromosome 3 in UMs as presented by the authors.

Manuel Jorge Rodrigues
David Gentien
Marc-Henri Stern
Laurence Desjardins
Jérôme Couturier

*Institut Curie
Paris, France*

Authors' Reply:

We are grateful for the opportunity to respond to the comments raised by Rodrigues et al. We presented detailed genomic analysis of 58 primary UM tissue specimens (a large cohort of exceptionally well characterized patient samples for such a rare tumor as UM) with clinical, histomorphological, and follow-up data using the SNP 6.0 whole genome microarray (Affymetrix).¹ The patient cohort contained genetically atypical variants of UM in the form of disomy 3 tumors that resulted in fatal liver metastases and monosomy 3 UMs with long-term patient survival exceeding 7 years. Our analyses revealed that amplification of *CNKSR3* and the resultant increase in protein expression were correlated with increased survival in patients with a monosomy 3 tumor (log rank, $P = 0.022$ and $P = 0.011$, respectively). These findings strongly suggest a functional role for *CNKSR3* in limiting the metastatic spread of a subset of monosomy 3 UM. Functional assessment of the protein and its influence on UM biology is underway.

We disagree with some of the points raised by Rodrigues et al. First, they state that several genome-scale studies

demonstrate that UMs have a relatively simple karyotype. From our own extensive experience of the gross chromosomal abnormalities present in UM, we agree that UMs have a much simpler karyotype than many solid tumors.^{8,9} However, unlike our study, the three studies cited by the authors to demonstrate this point did not evaluate individual gene copy number changes. Instead, they assessed loss or gain of whole chromosome arms or Giemsa bands within each chromosome arm.^{2,4,6} In 2010, we published our analysis of albeit a small number of snap-frozen UM samples ($n = 7$) that yielded high-quality DNA using the same SNP 6.0 assay.¹⁰ We reported that recurrent amplifications and deletions were present in UM samples at the resolution of single genes; this study formed the basis of the current project. To the best of our knowledge our own studies are unique in using genomic microarrays to identify pathogenic gene copy number changes in UM.^{1,10}

Second, Rodrigues and colleagues did not detect *CNKSR3* amplification in their comparative genomic hybridization analyses of 300 UMs. This is perhaps not surprising as our analyses identified *CNKSR3* amplification almost exclusively in a subset of monosomy 3 patients with more than 7 years' metastasis-free survival. It is therefore possible that the cohort examined by Rodrigues et al did not contain any such patients. However, since the microarray and survival data for the above-mentioned 300 cases, to the best of our knowledge, have not yet been submitted to a publically available database, it is not possible to test this hypothesis.

Rodrigues et al also postulate that the *CNKSR3* amplification reported in our study¹ could be a low-level amplification resulting from polysomy of 6q. However, we used a robust cut-off for amplification of >2.3 , as recommended by the Partek Genomics Suite (Partek, St. Louis, MO). Furthermore, the average copy number for *CNKSR3* amplification was 4.75. Importantly, we found no correlation between polysomy 6q and *CNKSR3* amplification (log rank, $P = 0.506$).¹

In contrast to the findings of Rodrigues et al, we found that quality control call rates were within the expected range for fixed tissue specimens, where DNA has some level of degradation compared to fresh or snap-frozen tissue samples, with a median of 90% (range, 87% to 91%).¹

Finally, we are fully aware of the cited publications of Jacobs et al¹¹ and Tuefford et al,^{12,13} who along with other investigators¹⁴ suggest that care should be taken in interpreting data from fixed tumor material. These studies also report good concordance between fixed and frozen tissues specimens when analyzed by genomic microarrays, provided that additional steps in both processing and analysis are incorporated. In analyzing our data using the Partek Genomics Suite, following the advice of an experienced bioinformatician (B.H.L.), we were able to incorporate the recommended analysis steps including use of the log2 of intensity values and smooth the data over 50 probe sets.

Most importantly, we also took the additional step of validating our SNP array data using immunohistochemistry,

establishing that amplification of *CNKSR3* correlated with increased protein expression in UM.¹ This key finding underpins our data, as not only have we identified a genetic biomarker of increased survival of patients with a monosomy 3 UM but also protein expression data suggest a potential biological role for *CNKSR3* in limiting metastatic spread.

Sarah L. Lake
Helen Kalirai
Andrew R. Dodson
Sarah E. Coupland
Bryony H. Lloyd

University of Liverpool
Liverpool, United Kingdom

Bertil E. Damato
Azzam F.G. Taktak

Royal Liverpool University Hospital
Liverpool, United Kingdom

References

1. Lake SL, Damato BE, Kalirai H, Dodson AR, Taktak AF, Lloyd BH, Coupland SE: Single nucleotide polymorphism array analysis of uveal melanomas reveals that amplification of *CNKSR3* is correlated with improved patient survival. *Am J Pathol* 2013, 182:678–687
2. Ehlers JP, Worley L, Onken MD, Harbour JW: Integrative genomic analysis of aneuploidy in uveal melanoma. *Clin Cancer Res* 2008, 14: 115–122
3. Harbour JW: The genetics of uveal melanoma: an emerging framework for targeted therapy. *Pigment Cell Melanoma Res* 2012, 25:171–181
4. Trolet J, Hupe P, Huon I, Lebigot I, Decraene C, Delattre O, Sastre-Garau X, Saule S, Thiery JP, Plancher C, Asselain B, Desjardins L, Mariani P, Piperno-Neumann S, Barillot E, Couturier J: Genomic profiling and identification of high-risk uveal melanoma by array CGH analysis of primary tumors and liver metastases. *Invest Ophthalmol Vis Sci* 2009, 50:2572–2580
5. Harbour JW, Roberson ED, Anbunathan H, Onken MD, Worley LA, Bowcock AM: Recurrent mutations at codon 625 of the splicing factor SF3B1 in uveal melanoma. *Nat Genet* 2013, 45:133–135
6. Hughes S, Damato BE, Giddings I, Hiscott PS, Humphreys J, Houlston RS: Microarray comparative genomic hybridisation analysis of intraocular uveal melanomas identifies distinctive imbalances associated with loss of chromosome 3. *Br J Cancer* 2005, 93: 1191–1196
7. Tuefford M, de Bondt A, Van den Wyngaert I, Talloen W, Gohlmann H: Microarray profiling of DNA extracted from FFPE tissues using SNP 6.0 Affymetrix platform. *Methods Mol Biol* 2011, 724:147–160
8. Damato B, Coupland SE: Genomic typing of uveal melanoma. *Arch Ophthalmol* 2009, 127:113–114. author reply 114–115
9. Damato B, Duke C, Coupland SE, Hiscott P, Smith PA, Campbell I, Douglas A, Howard P: Cytogenetics of uveal melanoma: a 7-year clinical experience. *Ophthalmol* 2007, 114:1925–1931
10. Lake SL, Coupland SE, Taktak AF, Damato BE: Whole-genome microarray detects deletions and loss of heterozygosity of chromosome 3 occurring exclusively in metastasizing uveal melanoma. *Invest Ophthalmol Vis Sci* 2010, 51:4884–4891
11. Jacobs S, Thompson ER, Nannya Y, Yamamoto G, Pillai R, Ogawa S, Bailey DK, Campbell IG: Genome-wide, high-resolution detection of copy number, loss of heterozygosity, and genotypes from formalin-

- fixed, paraffin-embedded tumor tissue using microarrays. *Cancer Res* 2007, 67:2544–2551
12. Tuefferd M, de Bondt A, Van den Wyngaert I, Talloen W, Gohlmann H: Microarray profiling of DNA extracted from FFPE tissues using SNP 6.0 affymetrix platform. *Methods Mol Biol* 2011, 724:147–160
 13. Tuefferd M, De Bondt A, Van Den Wyngaert I, Talloen W, Verbeke T, Carvalho B, Clevert DA, Alifano M, Raghavan N, Amaratunga D, Gohlmann H, Broet P, Camilleri-Broet S: Genome-wide copy number alterations detection in fresh frozen and matched FFPE samples using SNP 6.0 arrays. *Genes Chromosomes Cancer* 2008, 47:957–964
 14. Lips EH, Dierssen JW, van Eijk R, Oosting J, Eilers PH, Tollenaar RA, de Graaf EJ, van't Slot R, Wijmenga C, Morreau H, van Wezel T: Reliable high-throughput genotyping and loss-of-heterozygosity detection in formalin-fixed, paraffin-embedded tumors using single nucleotide polymorphism arrays. *Cancer Res* 2005, 65:10188–10191