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Response to Moskaluk *et al*

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To the Editor: It is with great interest that we read the comments by Moskaluk *et al*[1] in relation to our paper titled 'Identification of *c-kit* gene mutations in primary adenoid cystic carcinoma of the salivary gland' published in the October 2009 issue of *Modern Pathology*. We appreciate the comments from the authors, who are well-established investigators in the field. We understand the authors' concerns regarding the introduction of artifactual mutations using formalin-fixed paraffin-embedded tissue (FFPE);[2] however, as the study by Marchetti *et al* illustrates, these artifacts are likely to be observed when *very small amounts* of starting DNA (5 ng) are isolated and subjected to *multiple* PCR amplifications. As Marchetti *et al* point out, 'the occurrence of these artifacts can be prevented with the use of larger amounts of template DNA (at least 1 μg of DNA recovered from paraffin).' In our study, we measured the DNA content obtained after extraction to ensure that all tumor samples contained greater than 1.5 μg of starting template DNA. Moreover, tissue samples (which were obtained from large surgical resections and not small biopsies) were microdissected to minimize contamination from surrounding normal tissue and were subjected to a *single* PCR reaction, using a methodology similar to that outlined by Antonescu *et al*.[3]

Although the finding of more than one exon mutation in the *c-kit* gene is rare as Moskaluk *et al* note, ours is not the first study to report multiple *c-kit* mutations within the same tumor and loss or addition of mutations in primary versus metastatic tumor.[4,5] Multiple mutations were quite common in the series of Andersson *et al*, who utilized subcloning of PCR products for exons 12–15 and direct sequencing of exons 9–11 from fresh-frozen tissue of gastrointestinal stromal tumors (GISTs).[4] In this study, six of nine tumors with mutations in exons 11 had one or two additional mutations in exons 14 and 15. In addition, different mutations were found in the primary tumor versus secondary tumor, indicating the complexity between *c-kit* mutations and clinical behavior. These findings suggest the heterogeneity of tumor cells found within the same population and existence of subpopulations of tumor cells with different mutations. These subpopulations would not be detected by direct sequencing of PCR products, which requires the mutation subpopulation to consist of at least 15–20% of the total PCR product.

Although we cannot exclude with absolute certainty the introduction of artifactual mutations in this type of analysis, we believe these represent rare events. In fact, in our study, the majority of clones

sequenced for each tumor represented the wild-type sequence, with only a minority expressing point mutations. The majority of point mutations were found in exon 11, which is the most frequent exon mutated in the *c-kit* gene of GISTs. We expect that, if the mutations detected were due to artifacts caused by DNA damage that occurs during processing of FFPE tissue as Moskaluk *et al* suggest, we would have seen an equal distribution of mutations across all exons. Instead, we found the majority of point mutations clustering in the 5′ and 3′ regions of exon 11, with a minority of *c-kit* mutations occurring in exons 9, 13, and 17, as reported in GISTs. The finding of *c-kit* mutations in exact mutational hotspots as described in GISTs (ie, Leu576Phe, Val643Ala, Asn822Ser)[6–8] further adds to the validity of our results.

We agree with Moskaluk *et al* that published data from prospective clinical studies examining the role of anti-KIT-directed therapy have shown a very low response rate[9–13] and that therapeutic decisions should be made with caution. We recognize the limitations of our study, given our small sample size and lack of frozen-matched controls, which were not available for us to analyze at the time of this study. We hope that our paper would encourage further examination of the *c-kit* gene in adenoid cystic carcinomas of the salivary gland (ACCSG) using better sequencing approaches, such as subcloning or 454 sequencing; otherwise, we may miss small subpopulations of *c-kit* mutations in tumor samples.

We have acknowledged in our conclusions in the paper that future studies in a larger series are needed to determine the prognostic and therapeutic implications of *c-kit* mutations in ACCSG. We have begun our efforts to collect frozen tumor and fresh cell lines of ACCSG for further study, and hope that we can collaborate with other centers in these efforts.

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