

Correspondence

Clonal Origin and Expansions in Neoplasms: Biologic and Technical Aspects Must Be Considered Together

To the Editor-in-Chief:

Microsatellite-based clonality assays include the analysis of X-chromosome inactivation (XCI) and loss of heterozygosity (LOH) of tumor suppressor genes, and have been rarely applied to differentiate clonal origin from clonal expansion in neoplasms. The key elements for that distinction are: tumor natural history with particular attention to the relative timing between test conversion and clonal expansion, the lesion cell kinetic, and sample conditions. Studies based on allele ratio of genes involved in the transformation pathway must validate technique conditions to obtain reliable quantification methods able to detect clonal growths. These aspects are relevant and, probably due to space restrictions, have not been considered in detail in a recent paper on the clonality of in-transit melanoma metastasis.¹

Although clonality is considered the hallmark of neoplasms, the distinction between clonal origin and clonal expansion in tumors remains controversial. *A priori*, a monoclonal proliferation is assumed to be neoplastic, whereas a polyclonal lesion is thought to be reactive. However, there are many exceptions to this rule. Additionally, there is no consensus on the application of clonality markers. Clonality analysis has been used to test malignant transformation and tumor progression,^{2,3} but the results must always be interpreted in view of the natural history of the neoplasm. The relationship between the molecular marker and the pathway of neoplastic transformation is essential, in particular, the relative timing between the positive conversion of the marker and the clonal expansion. Clonality results will support a clonal origin only if the clonal expansion occurs after the positive conversion (Figure 1). Positive conversions taking place after the clonal expansion will result in heterogeneous marker patterns, which do not support clonal origin.⁴ This is a key element for studies based on the analysis of tumor suppressor genes, especially if reduced number of cells (microdissected samples) are used.

Transformed cells result in neoplasms if genetically damaged cells are able to expand clonally. In contrast, extensive genetic damage triggering cell apoptosis will not result in neoplasms. Therefore, it is artificial to separate the analysis of tumor clonality and cell kinetic (proliferation/apoptosis), as demonstrated by the close relationship between them in benign adrenal cortical proliferative lesions.⁵ Expanding clones would also sug-

gest that somatic genetic alterations contribute to the kinetic advantage of those cells, which eventually outnumber other cells and result in monoclonal patterns. In that sense, clonality would be the by-product of tumor cell selection, especially for advanced neoplasms, and one of the first alterations in early neoplasms as well (Figure 1). These situations are highlighted by LOH analysis of tumor suppressor genes (advanced neoplasms) and XCI assays (early neoplasms). This combined analysis of clonality and cell kinetics better defines the evolution and progression of neoplasms.^{2,3,5-8} LOH analysis of tumor suppressor genes in a given tumor will inform on clonal origin only if concordant patterns with several genetic markers are demonstrated.^{4,8,9} The interpretation must consider that true monoclonal lesions retain the constitutional heterozygosity before the conversion point and that tumor heterogeneity and progressive cell selection can result in discordant microsatellite patterns in samples from different areas within a single tumor (intra-tumoral heterogeneity).^{6,10,11} If the genetic abnormalities determine a kinetic advantage, tumor cells revealing LOH will overgrow and become the predominant genotype (clonal expansion, Figure 1).

Among sample conditions, the size is the most important limiting factor leading to misinterpretations due to tumor heterogeneity.^{3,7} Microdissection techniques allow very selective and homogeneous cell samples, but the sampling might not be representative of the tumor. Firstly, small cell groups descended from a common progenitor may grow together like a clone (patch size concept), which can explain monoclonal patterns in small-sized samples. Secondly, sample cells must be representative of tumor features (eg, kinetic and invasive capacities). If clonality is not evaluated in the proper biological context, the results might be confusing or have unknown clinical meaning. A typical example of this situation is microheterogeneity in tumors that tend to give disparate results whose meaning remains unknown. Only multiple samplings of enough size (~100 cells) from different tumor areas and running tests in duplicate can avoid this problem; this protocol should be systematically done before accepting the results as relevant.

The intratumoral heterogeneity and the heterogeneous cell composition of solid tumors make the quantitative determination of the allele ratio (proportion of each allele in samples normalized by the corresponding control tissue) an absolute requirement to prove any clonal origin or expansion. Allele ratios greater than 3:1 or 4:1 in normalized samples are considered evidence of monoclonal proliferation.^{3-8,12} This thresh-

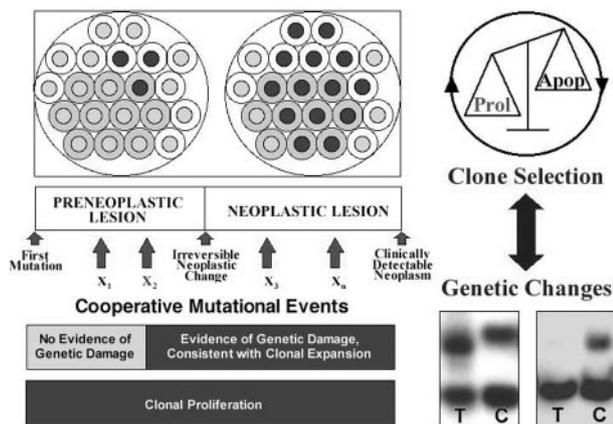


Figure 1. Cell kinetics and genetic changes during the clonal evolution of neoplasms. The first genetic abnormality during the neoplastic transformation is assumed to induce a clonal proliferation, the hallmark of neoplasms (bottom, left-hand bar). However, any other genetic changes will detect the lesion after the marker conversion only (eg, microsatellite instability or LOH, X₂ in the diagram). Tumor tissue heterogeneity complicates the detection because results supportive of monoclonal proliferation (right cell field) will be obtained only if the abnormal cells (dark nuclei) are prevalent in the sample (gray cytoplasm). The expansion of genetically damaged cells (clone selection) is always due to disbalanced kinetic (↑ proliferation and/or ↓ apoptosis).

old means concordant allele patterns for a given marker in 75% to 80% of the sample cells. However, reliable results require keeping linearity of the allele ratio between the amplified product and the target DNA of the sample. For that purpose, technical aspects of the PCR amplification are essential. The reaction should be maintained in the exponential phase avoiding the plateau (product saturation) and the amount of tissue in control and test samples should be similar, thus correcting biased patterns due to artifacts induced by small target concentrations in tumor samples only.³ In addition, microdissected samples normally show a high incidence of PCR artifacts due to the small concentration of target DNA, fixation-induced changes of DNA, and conditions in the amplification of repetitive sequence (especially for those CG-rich sequences) favoring misannealing and hairpin formation.¹³ Appropriate modifications must be established to avoid these problems, thus improving the reproducibility of LOH and MSI test in microdissected samples.

In conclusion, a proper interpretation of clonality tests requires a combined knowledge of the tumor natural history and technical aspects. Using the spectrum of clonality tests available, a reliable distinction of clonal origin/expansion can be made considering the relative timing between test conversion and clonal expansion, the lesion cell kinetic, sample conditions, and the conditions for the allele ratio determination.

Lucia Pozo-Garcia

Homerton University Hospital
 London, United Kingdom

Salvador J. Diaz-Cano

Barts and the London School of
 Medicine and Dentistry
 London, United Kingdom

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Authors' Reply:

We appreciate the comments from the authors' letter and would like to respond by stating that the general purpose of this manuscript was to evaluate the genetic heterogeneity among in-transit melanoma metastasis assessing microsatellites with loss of heterozygosity (LOH), an established methodology.¹ In-transit melanoma is a rare phenomenon often occurring after removal of a primary tumor cutaneous melanoma tumor. This disease manifestation is clinically evident from where the site of origin existed as opposed to head and neck cancers where the primary may be unknown or Barrett's esophagus where LOH has been used for clonal origin and delimiting field cancerization with clonal expansion.²⁻⁴ Thus, our intent was to assess those LOH events associated with in-transit metastasis with further interest to determine

whether a pattern of heterogeneity existed for the panel of markers assessed. Furthermore, intratumoral heterogeneity and congruity with the primary tumor was evaluated for concordance.

Multiple polymorphic microsatellite markers were chosen for their informativity and frequency in melanoma tumors. In addition, many tumors were assessed to allow for sufficient number of LOH events to occur to avoid a conclusion of homogeneity based on retention alone or heterogeneity due to nonlinear random occurrences.⁵ To further determine whether results were consistent, intratumor heterogeneity was assessed. However, to avoid false monoclonality interpretation due to inadequate sampling from "patch size" clones, three separate regions were chosen which were widely spaced and randomly selected.⁶ Additionally, a large enough sample was microdissected from each specimen to ensure a sufficient number of cells and DNA quantitated for conformity. Finally, primary tumor blocks were assessed in a similar fashion to confirm the findings with concordant patterns of the genetic markers assessed. The fact that consistency was demonstrated through all three aspects of the investigation confirms the reliability of the methodology in this study and leaves little doubt that these results occurred randomly for this specific disease entity.

We agree that good laboratory practice under rigorous standard operating procedures must be strictly adhered to for any laboratory that is assessing LOH. Optimal sample conditions and repetitive assessments should be routine for accurately assessing allelic imbalances (AI). As the biology and relevance of these AI is still developing, optimal standardization has not been consistent in the literature. Assessment for clonality in tumor specimens using assays previously reported as the authors suggest does have limitations in interpretation. With regard to assessing molecular markers in relation to a tumor's natural history, all of the lesions were of the in-transit type, which is a unique model for this field of investigation. However, an inherent problem with any study evaluating patients' tumors is the inability to collect all specimens at identical time points in the disease progression spectrum. The accumulation of genetic alterations is a continuum for each individual tumor cell and thus one can never obtain sufficient number of specimens all at the same time point during neoplastic transformation and progression to make an absolute conclusion with certainty. We recognize the authors' concern regarding timing between test conversion and clonal expansion as well as lesion cell kinetics but this method provides the most clinically relevant approach for evaluating the unique pathology and biology of in-transit melanoma disease. In-transit recurrence is consistent with dormant tumor cell clones from the primary tumor trapped in intervening lymphatics and our findings provide a genetic association for this clinical experience.

Bret Taback
Dave S.B. Hoon

John Wayne Cancer Institute
Santa Monica, California

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Interleukin-3 Receptors in Hodgkin's Disease

To the Editor-in-Chief:

Growing evidence suggests that deregulated apoptosis is a frequent occurrence in a variety of human malignancies.¹ The tumor cells in classical Hodgkin's disease (HD), historically named Hodgkin and Reed-Sternberg (HRS) cells, derive from germinal center B cells and often contain "crippling" somatic mutations within rearranged immunoglobulin (Ig) heavy chain genes.² Because such "crippling" mutations trigger apoptosis in germinal center B cells, their detection in HRS tumor cells indicate the presence of survival factors other than surface Ig.

In the February 2002 issue of *The American Journal of Pathology*, Aldinucci et al³ reported on the expression of interleukin-3 receptors (IL-3R) in HRS cells. As the authors pointed out correctly, it is quite surprising that IL-3R expression has not yet been investigated in the context of HD because of the ligand for this receptor, IL-3, also called multicolony-stimulating factor, is probably one of the least restricted growth factors, exerting its effects on hemopoietic stem cells and progenitors of numerous lineages,⁴ including the lymphoid lineage, as shown by *in vitro* differentiation of IL-3-dependent B-cell precursors into mature B cells.⁵ In addition to describing IL-3R expression in HRS cells, Aldinucci et al³ examined a number of HD-derived cell lines to address the functionality of these receptors. Among the HD cell lines tested, IL-3R expression levels showed a remarkable variability. However, the increased growth rates of cultured HD cells on stimulation with IL-3 did not strictly reflect the differences in IL-3R expression. The fact that L1236 cells, which express low levels of IL-3R, had a stronger response than any other HD cell line to exogenous IL-3, should remind us to interpret data obtained from cultured HD cells with great caution. The relatively minor growth response of the