Consultations in Molecular Diagnostics

Distinguishing *de Novo* Second Cancer Formation from Tumor Recurrence

Mutational Fingerprinting by Microdissection Genotyping

Raj Rolston, Eizaburo Sasatomi, Jennifer Hunt,
Patricia A. Swalsky, and Sydney D. Finkelstein

*From the Division of Anatomic Pathology, Department of
Pathology, University of Pittsburgh Medical Center,
Pittsburgh, Pennsylvania*

Patients with one form of cancer are known to possess a higher risk for development of a second tumor, presenting synchronously or metachronously over time. Distinguishing whether the second tumor represents a *de novo* cancer or a recurrence/metastasis of the first cancer has important implications for treatment and prognosis and specific clinical and pathological evidence is sought to resolve this issue. *De novo* cancer formation is favored by long latency interval (usually exceeding five years), better differentiation in the subsequent tumor, solitary second tumor formation and occurrence of the later tumor in a site not typical of metastatic spread. Conversely, recurrent metastatic disease is favored by short interval to second cancer formation, similar histology with increased anaplasia in the second tumor and lack of *in situ* malignancy and multifocal tumor deposits. While these criteria are sufficient in most instances to distinguish between the two, varying degrees of uncertainty can persist with a minority of cases remaining unresolved.

Immunohistochemistry can be useful if unique staining similarities or differences between tumors can be demonstrated, as when the second neoplasm is derived from a different cellular histogenesis such as a sarcoma with epithelioid growth characteristics *versus* a poorly differentiated carcinoma. Unfortunately, second primary tumors often fall into the squamous cell carcinoma or adenocarcinoma cell groups and can be expected to have similar immunohistochemical characteristics. This is especially the case when a field of cancer susceptibility exists as in the case of head, neck, and lung squamous cell carcinomas, multifocal adenocarcinoma of the large intestine, or transitional cell carcinomas of the urinary tract.

Human malignancy arises, not from a single genetic alteration, but by a process of stochastic acquisition of cancer-related genetic damage over time.¹⁻³ Even in the context of cancer susceptibility associated with inherited, environmental, or dietary factors, and in the patient with two independent tumors, clonal evolution involving individual cancer cells selected on the basis of greater expression of malignant phenotypic characteristics gives a unique pattern of mutational alteration to each individual tumor. While different topographic areas within a single tumor may exhibit differences in the overall profile of acquired molecular damage, recurrent and/or metastatic tumors may be expected to share most, if not all, of the genotypic profile of mutational alterations in the primary tumor from which they were derived.^{4,5} In contrast, *de novo* primary tumors may be expected to manifest significant differences in acquired somatic mutations giving each a unique fingerprint of gene damage. A broad panel of gene alterations, which can provide the necessary scope to define critical differences in genotypic profile, is central to genetic analysis aimed at determining whether a second tumor is due to recurrence/metastasis or a *de novo* second tumor.

We have developed a high throughput system of mutational analysis for this purpose. The system is based on two fundamental methodologies: tissue microdissection to sample tumors at sites representative of their greatest cellular aggressiveness and genotyping for allelic loss to give sensitive, simple, and cost-effective mutational analysis for detailed comparative mutational fingerprint analysis. The case described illustrates the efficacy of this approach.

A 58-year-old white male underwent colonic resection for a moderately differentiated adenocarcinoma of the colon with invasion through the muscularis propria into the pericolic fat. One pericolic lymph node was pos-

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Address requests for reprints to Sydney D. Finkelstein, M.D., Department of Pathology, University of Pittsburgh Medical Center, 200 Lothrop Street, PUH A610.2, Pittsburgh PA 15213. E-mail: finkelsteind@msx.upmc.edu.

Figure 2. Allelic imbalance analysis. Capillary electrophoresis of fluorescently labeled microsatellite PCR products. The criteria for conservative threshold determination of allelic loss was defined as a ratio between informative allelic band heights of less than 0.5 or greater than 2.0 (positive allelic loss). Note the presence of two near equivalent allelic peak heights in the normal microdissected tissue sample indicating both informativeness for the particular marker and the presence of a balanced PCR reaction without artificial induction of allelic loss. An example of a non-informative marker is shown. Note that multiple samples may be run simultaneously for multiplex analysis.

itive for metastatic adenocarcinoma. The tumor stage was T3/N1/M0. Five years later, anemia and occult blood in stools were noted. Radiological studies revealed a small a bowel mass that was confirmed endoscopically. A moderately differentiated adenocarcinoma was found involving the mucosa of the second portion of the duodenum. While metastasis from the colonic adenocarcinoma could not be ruled out, *de novo* primary tumor was preferred, given the long latency interval of five years and the unusual location for colonic metastasis to the small intestine. The issue remained unresolved after thorough clinical, physical, laboratory, and pathological analysis of representative tissue specimens. Histochemical and immunohistochemical evaluation could not discriminate between primary versus metastatic disease. To address this issue, microdissection genotyping, using a broad panel of adenocarcinoma associated microsatellite markers for allelic loss (loss of heterozygosity), was applied to discrete sites in tumor tissue to define the profile of allelic loss.

Four serial, unstained sections, four microns in thickness, of formalin-fixed paraffin-embedded tissue provided the basis for mutational analysis.⁶ In addition to the non-neoplastic tissue sample, three tissue targets were microdissected (Figure 1): the colonic tumor at the point of deepest invasion, the pericolonic lymph node metastasis, and the small intestinal adenocarcinoma at the deepest point of invasion. Noteworthy was the pattern of pericolonic lymph node metastasis (Figure 1, B and C). The metastasis at this site was largely necrotic with only

Figure 1. Topographic microdissection. **A:** The primary colonic cancer has been sampled at the point of deepest invasion (arrow). **B** and **C:** A sample has also been obtained from a pericolonic lymph node metastasis subjacent to the same tumor seen at low power (**B**) and at high power (**C**). The metastatic tumor in this case was highly necrotic consisting only of a rim of viable tumor at the periphery. Thus microdissection took the form of a thin doughnut of viable tumor (arrow). Also note the minute amount of tissue required to enable detailed mutational profiling (third section has not been microdissected). A representative sample of non-neoplastic, normal appearing colonic mucosa has also been taken. In a similar fashion, the small intestinal tumor was microdissected to provide representative material for mutational fingerprinting.

PHS01-9594 JACOBS	1p MYCL	3p26 D3S 1539	3p26 D3S 2303	5q21 D5S 592	5q21 MCC	9p21 D9S 254	9p21 D9S 251	10q23 D10S 520	10q23 D10S 1173	10q23 MX11	17p13 D17S 974	17p13 D17S 1289	17p13 D17S 1303
NORMAL COLON MUCOSA	I	I	I	I	NI	I	NI	I	I	I	I	I	I
COLON TUMOR 96-10688	LOH T	LOH B	LOH T	LOH B	NI	NO LOH	NI	LOH T	NO LOH	NO LOH	LOH B	NO LOH	NO LOH
LN MET 96-10688	LOH T	LOH T	LOH B	LOH B	NI	NO LOH	NI	LOH T	NO LOH	LOH T	LOH B	LOH T	LOH T
COLON TUMOR 01-3965	LOH T	LOH T	LOH B	LOH B	NI	NO LOH	NI	LOH T	LOH B	LOH T	LOH B	LOH T	LOH T

Figure 3. Microdissection genotyping. I: informative; NI: noninformative; NO LOH: no loss of heterozygosity (allelic balance); LOH: allelic loss. Initial allelic loss alteration is indicated in dark gray, second allelic loss event involving the same microsatellite is indicated in the four light gray boxes.

a thin rim of viable metastatic tumor present at the periphery of the deposit.

Under stereo-microscopic guidance using an Olympus 52-STs microscope, the defined regions of tissue were manually removed from each of the sections using a scalpel. The tissue was collected in 50 μ l of buffer (Tris-HCL pH 7.0), treated with proteinase K (10 mg/ml), boiled for 5 minutes and stored at -20°C until ready for genotyping. Aliquots of the extracted nucleic acid underwent polymerase chain reaction (PCR) amplification in individual PCR reactions for a broad panel of 13 microsatellite markers situated in proximity to known tumor suppressor genes (Figure 2) located in genomic regions corresponding to 1p34, 3p26, 5q21, 9p21, 10q23, and 17p13. These are sites of known tumor suppressor genes including *VHL*, *OGG1*, *APC*, *MCC*, *CDKN2A*, *PTEN*, *MX11*, *DMBT1*, and *p53*. Analysis of allelic loss involved the use of fluorescently labeled primers (HEX, TET, FAM, NED, Applied Biosystems, Foster City, CA) designed to flank each of the microsatellites. Non-neoplastic, normal appearing tissue was microdissected to establish the polymorphic status and as an internal control for tissue fixation effect on DNA and to obtain a PCR reaction with allelic balance free from allelic drop out. Only microsatellites showing two balanced alleles from normal microdissected tissue samples were evaluated for allelic loss in corresponding tumor samples (Figure 2). Tissue samples exhibiting single peaks in non-neoplastic tissue were designated non-informative (Figures 2 and 3).

Two of 13 microsatellite markers were found to be non-informative, one situated at 5q21 and a second at 9p21 (Figure 3). Of the remaining 11 informative microsatellite markers, six manifested allelic loss in the colonic adenocarcinoma. The pericolic lymph node metastasis displayed nine allelic loss alterations of which four were identical in allele involvement with that of the primary colonic tumor from which it had arisen. Noteworthy were two allelic loss events which affected the same two markers for 3p26 (D3S1539 and D3S2303) but which led to loss of the opposite alleles to that in the primary tumor. Given that the pericolic lymph node metastasis was derived from the associated colonic tumor, the temporal progression of allelic loss acquisition can be described

as in Figure 4. Initially the colonic adenocarcinoma acquired the shared allelic loss alterations seen in both the primary colon cancer and its metastasis. Metastatic seeding occurred at that time leading to accumulation of new allelic loss alterations in the pericolic lymph node metastasis. These new events in the pericolic lymph node metastasis affected the same alleles subsequently altered in the primary colon cancer. The small intestinal tumor revealed 10 allelic loss alterations of which nine were not only identical with respect to the specific markers involved but also with respect to the specific alleles which had been lost (Figure 3). The fingerprint of the pericolic lymph node metastasis and the subsequent small intestinal adenocarcinoma were very similar, differing only with respect to a single additional allelic loss alteration in the small bowel tumor. These genotypic features support the concept of a single colonic adenocarcinoma with small bowel metastasis after a five-year latency period.

Figure 4. Temporal acquisition of mutational change. Initial shared allelic loss alterations are seen in both the primary colon cancer and lymph node metastasis. Metastatic seeding occurring at that time led to the accumulation of new allelic loss alterations in the lymph node metastasis which affected the same alleles subsequently altered in the primary colon cancer. The small intestinal tumor occurring five years later showed nine allelic loss alterations identical to that seen in the lymph node metastasis and one additional, new allelic loss.

This case illustrates the practical difficulty of dealing with the presence of a second malignancy in a patient known to have had a previous cancer. Cogent arguments, based on clinical and histological criteria, may be made for the diagnosis of *de novo* tumor formation or for tumor recurrence/metastasis. Either of these could be contrary to that ultimately established by definitive mutational genotyping. Given the availability of techniques described in this report, the distinction can be performed in an objective manner using methods that are simple, high throughput, and cost effective.

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