

## PEPTIDE NUCLEIC ACID CLAMP PCR: A NOVEL K-*ras* MUTATION DETECTION ASSAY FOR COLORECTAL CANCER MICROMETASTASES IN LYMPH NODES

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**Inaccurate staging of colorectal cancer (CRC) has been attributed to the failure to detect lymph node metastases by conventional pathology. We have previously reported the use of lymphatic mapping to accurately identify those lymph nodes most likely to harbor micrometastatic disease and permit focused pathologic examination. Mutation of K-*ras* allele at codons 12 or 13 occurs frequently in early stages of CRC development. The purpose of our study was to assess sentinel lymph nodes (SLN) for occult CRC micrometastases using a unique peptide nucleic acid (PNA) clamp PCR assay specific for K-*ras* mutations. Seventy-two paraffin-embedded primary CRC and paired SLN were evaluated by PNA clamp PCR for K-*ras* mutations. Thirty primary tumors (42%) were positive for K-*ras* mutations, and in 5 of these cases the SLN were positive for metastases by Hematoxylin and Eosin staining. PNA clamp PCR identified occult metastases in an additional 6 patients, upstaging 24% of K-*ras* positive primary CRCs ( $p = 0.014$ ). No K-*ras* mutations were detected among the 20 noncancer lymph nodes assessed. This study demonstrates the utility, specificity and sensitivity of PNA clamp PCR assay in identifying occult micrometastases in the SLN of CRC patients by single-base mutation analysis.**

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**Key words:** colorectal cancer; sentinel lymph node; metastases, K-*ras* mutation; peptide nucleic acid

K-*ras*, an oncogene mutation often found in adenomas, is consistently associated with CRC progression. The identification of K-*ras* mutation in primary tumors and/or subsequent metastasis may provide prognostic insight into a particular tumor's biology and early disease progression.<sup>1,2</sup> Mutations of the K-*ras* allele at codons 12 and 13 occur frequently at early stages of CRC development.<sup>3</sup> Because this genetic alteration has been shown to demonstrate stability during disease progression, the detection of K-*ras* mutation maybe used to identify metastases associated with primary tumors that contain this specific genotypic aberration.<sup>4–7</sup>

The presence of lymph node metastases is the single most important prognostic factor in patients diagnosed with early stage CRC. The 5-year survival rate in patients with stage I or II disease exceeds 80%,<sup>8</sup> but once lymph node metastases are present, the 5-year survival rate is reduced to 50–60%.<sup>8,9</sup> A variety of approaches have been proposed to improve the accuracy of staging the tumor-draining lymphatic basin,<sup>10–13</sup> and it has been suggested that for accurate staging of CRC, a minimum number of lymph nodes must be obtained for histopathologic examination.<sup>14</sup> However, pathologic processing and histologic assessment of multiple lymph nodes from a CRC resection is rather cumbersome, time consuming and costly. Furthermore, reports have found conventional H&E to miss occult metastatic tumor cells during lymph node evaluation.<sup>15–18</sup> More sensitive, accurate and rapid techniques/assays are needed to improve the current diagnostic approach.

The operative technique of lymphatic mapping and SLN identification has been shown to accurately reflect the pathologic status of the tumor-draining nodal basin for a variety of solid neo-

plasms.<sup>19–21</sup> This approach provides a more cost-effective and focused analysis of those lymph nodes most likely to harbor occult metastases associated with early stage disease.<sup>22</sup> Immunohistochemistry (IHC) has demonstrated improved sensitivity for detecting lymph node micrometastases by upstaging an additional 20–40% of CRC patients.<sup>15–18</sup> The clinical implications of these findings remain controversial, possibly due to the fact that the results are based on morphology as well as limitations associated with antibody specificities.<sup>15,16,18</sup> However, a molecular assay for detecting K-*ras* mutation could be useful in identifying SLN micrometastases of those primaries with K-*ras* mutation.

Although genotypic PCR assays can amplify specific nucleic acid targets for detection, it is often used in conjunction with sequencing procedures for the detection of single-base mutation such as K-*ras*. It is very difficult to accurately detect the scarce copies of single-base mutated gene(s) of occult tumor cells among thousands of copies of wild-type DNA (wtDNA) contained in normal tissues. To date, there is very limited success in detecting single-base mutation of occult tumor cells in lymph nodes using DNA PCR assays. In our study, we have developed a unique and sensitive assay that detects specific K-*ras* mutations in CRC. To successfully target the mutated DNA, we designed a PNA oligo as a clamp to inhibit wtDNA from competing in the PCR as a template. PNAs are synthetic oligonucleotides with peptide backbones that replace sugar groups.<sup>23,24</sup> The PNA probe recognizes and binds to complementary DNA genomic template sequences with a higher affinity and specificity than DNA-DNA binding. The unique binding property and the inability to extend like a primer make PNA the ideal clamp to inhibit PCR of the wtDNA and allow amplification of the unobstructed K-*ras* mutant template. This approach has proven quite sensitive for identification of K-*ras* mutations in tumor specimens<sup>25</sup> but has yet to be assessed in conjunction with more modern surgical techniques such as sentinel lymph node mapping for the detection of occult tumor cells asso-

**Abbreviations:** AJCC, American Joint Committee on Cancer; CRC, colorectal cancer; ECL, electrochemiluminescence; H&E, Hematoxylin & Eosin staining; IHC, immunohistochemistry; K-*ras*, Kirsten ras mutation; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PNA, peptide nucleic acid; SLN, sentinel lymph node; TBR, Tris (2,2'-bipyridine) ruthenium(II); wtDNA, wild-type DNA.

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ciated with the earliest spread of CRC. The purpose of our study was to determine whether the use of PNA clamp in PCR can successfully detect presence of *K-ras* mutation(s) in primary tumors to provide a selective target for the molecular assessment of SLN micrometastases in patients with early-stage CRC.

#### MATERIAL AND METHODS

##### Patient tumor samples

Seventy-two paraffin-embedded primary tumors and paired SLN from CRC patients treated at St. John's Health Center (Santa Monica, CA), McLaren Regional Medical Center (Flint, MI) and Century City Hospital (Los Angeles, CA) were evaluated (Table I). The average patient age was 72 years (range: 37 to 97 years). Thirty-five patients were female and 37 were males. Mean tumor size was 4.1 cm (range: 0.2 to 8 cm). Location of the primary tumor was as follows: right colon 25, transverse colon 12, left colon 24 and rectum 11. Twenty-five patients were American Joint Committee on Cancer (AJCC) stage I, 21 patients were AJCC stage II and 26 patients were AJCC stage III. A mean of 2 SLN was identified from each patient (range: 1 to 8 SLN).

Primary tumor specimens and non-SLN were processed for routine microscopic analysis in a standard manner. Each SLN, noted blue intraoperatively by the surgeon, was submitted in its entirety or bisected in half along its greatest axis, depending on its size, for pathologic processing. Two 4  $\mu$ m sections of the paraffin-embedded SLN were cut at 2 levels separated by 200  $\mu$ m. One section from each level was stained with H&E and another with cytokeratin IHC using the AE-1/AE-3 cytokeratin antibody cocktail (Dako Corporation, Carpinteria, CA). IHC stained slides were interpreted according to strict histologic criteria that required strong immunoreactivity combined with microanatomic and cytologic features compatible with CRC.

##### DNA isolation from cell lines and paraffin-embedded tissues

Established cell lines with known *K-ras* mutation (colon cancer: SW480, GGT→GTT and LoVo, GGC→GAC; pancreas cancer: PANC-1, GGT→GAT and MIA PaCa-2, GGT→TGT) and cell lines containing wtDNA (pancreas cancer: BxPC-3; melanoma: MA, MB and MC; and breast cancer: MCF-7) were cultured in RPMI 1640 or Dulbecco's Modified Eagle Medium culture media (Gibco Life Sciences, BRL, Grand Island, NY) with 10% fetal bovine serum (Gemini Bio-Products, Woodland Hills, CA), Streptomycin (Gibco Life Sciences) and incubated in 5% CO<sub>2</sub>. Adher-

ent cells were washed with phosphate-buffered saline (PBS), harvested in 0.05% trypsin (Gibco Life Sciences), counted and assessed for viability by trypan blue exclusion. DNA was isolated from the cell lines using DNAzol (Molecular Research Center, Inc., Cincinnati, OH).

An additional 10 10  $\mu$ m-thick sections were cut from each paraffin-embedded tissue for DNA isolation. Paraffin tissue sections were deparaffinized with xylene for 5 min, washed once with 100% ethanol and dried. DNA was isolated from the dissected tissue using QIAamp Tissue Kit (QIAGEN, Hilden, Germany) and quantified using UV spectrophotometry. DNA from paraffin-embedded normal colon tissues from 10 patients was isolated similarly.

##### PNA clamp directed PCR

Biotinylated oligonucleotide sense primer and tris (2,2'-bipyridine) ruthenium(II) (TBR)-labeled oligonucleotide antisense primer for PCR were synthesized by The Midland Certified Reagent Company (Midland, TX). PNA clamps were synthesized from Perkin Elmer Biosystems (Framingham, MA). Genomic DNA extracted from cell lines and paraffin-embedded tissues was amplified using PCR in a 20  $\mu$ l reaction containing 16 mM Tris-HCl (pH ~8.3), 80 mM KCl, 2.4 mM MgCl<sub>2</sub>, 800  $\mu$ M dNTPs, 75 nM *K-ras* sense primer, 75 nM *K-ras* antisense primer, 400 nM PNA, 0.5 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Biosystems) and 20 ng of genomic DNA. PCR thermocycling after a 12 min enzyme activation at 94°C was as follows: 60 sec at 94°C, 50 sec at 70°C, 50 sec at 58°C and 60 sec at 72°C for a total of 40 cycles followed by a 7 min final extension at 72°C. In each experiment, cell lines verified to contain mutant *K-ras*, normal colon tissue and noncancer lymph nodes, and PCR reagents without template were run in parallel as positive, negative and no template controls, respectively.

The PNA clamp was designed to hybridize complimentary to the wild-type *K-ras* allele surrounding codon 12 and 13.<sup>25</sup> PCR was performed using a biotin labeled sense primer, 5'-(Biotin)-ATCGT-CAAGGCACTCTTGCTAC-3', a TBR labeled antisense primer, 5'-(TBR)-GTACTGGTGGAGTATTTGATAGTG-3' and PNA clamp, H<sub>2</sub>N-TACGCCACCAG CTCC-CON<sub>2</sub>N. PNA hybridizes to the wtDNA template securely inhibiting annealing of the partially overlapping reverse primer and inhibit the amplification of the wtDNA at the *K-ras* allele (Fig. 1). As for any of the *K-ras* mutant allele variants at codons 12 and 13, the PNA/DNA hybrid is unstable due to the base pair mismatch and therefore does not inhibit the *Taq* polymerase from extending the reverse primer on the mutated tumor DNA.

TABLE I - PATIENT DEMOGRAPHICS

Patient characteristics	Study population (n = 72)
Gender	
F	35
M	37
Age	
Mean	72
Range	(37-97)
Primary site	
Right colon	25
Transverse colon	12
Left colon	24
Rectum	11
AJCC stage	
I	25
II	21
III	26
Tumor size	
Mean	4.1 cm
Range	(0.2-11 cm)
Number of SLN	
Mean	2.2
Range	(1-8)
Number of non-SLN	
Mean	11.5
Range	0-36

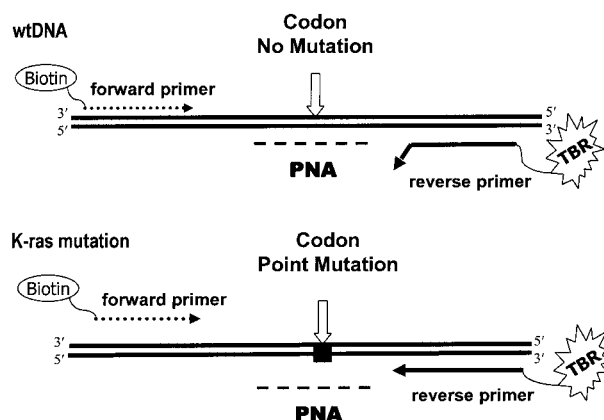


FIGURE 1 - Schematic pictorial of PNA clamp directed PCR. Upper figure denotes PNA/wtDNA complex, no amplification. Lower figure demonstrates amplification of DNA template containing *K-ras* mutation.

### Gel electrophoresis and electrochemiluminescence detection

PCR products (5  $\mu$ l) were electrophoresed on a 2% agarose gel to verify the presence of amplified product of the expected size. For the electrochemiluminescence (ECL) assay, post-PCR products (5  $\mu$ l) were incubated in 50  $\mu$ l of M280 streptavidin coated Dynabeads® (0.125 mg/ml) (DynaL Biotech, Lake Success, NY) for 30 min at room temperature with constant vortexing followed by the addition of 300  $\mu$ l of ORIGEN® Assay Buffer prior to starting the Origen® Analyzer. The Origen® Analyzer (IGEN, International, Gaithersburg, MD) measures the ECL activity generated by the electrochemical oxidation reaction of the TBR and assay buffer as previously described.<sup>26</sup> At least 3 paraffin-embedded normal colon tissue DNA samples were incorporated throughout each PCR/ECL assay to determine the background ECL level and as a reference control. Samples were considered positive if the ECL level detected was greater than 2 standard deviations above the mean of ECL level of the paraffin-embedded normal colon tissue DNA samples (cut-off point). The assay was optimized and the cut-off point is determined using the controls included in each assay. Assays were repeated at least 2 times to verify the results.

### Sequencing analysis

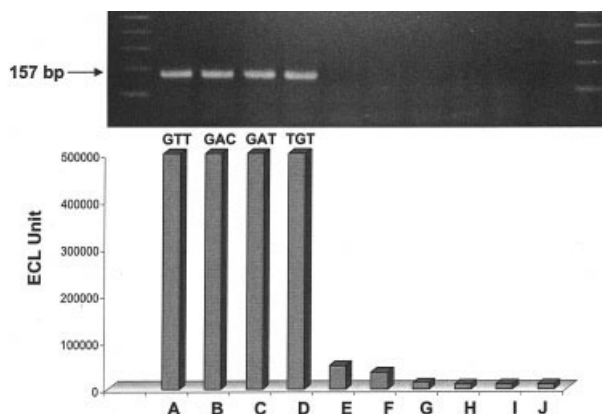
Sixteen K-ras mutation positive samples and 17 K-ras mutation negative samples from primary tumors were analyzed by sequencing. DNA (~10 ng) was amplified using PCR in a 50  $\mu$ l reaction volume containing 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 800  $\mu$ M dNTPs, 75 nM K-ras forward primer, 75 nM K-ras reverse primer and 1.25 U of AmpliTaq Gold DNA polymerase (Perkin Elmer, Norwalk, CT). Thermocycling steps were 30 sec at 94°C, 30 sec at 50°C and 30 sec at 72°C for a total of 35 cycles followed by a 7 min final extension at 72°C with a 12 min at 94°C step before the first cycle. PCR products were electrophoresed on a 2% agarose gel and the DNA fragment was excised and purified using the QIAquick Gel Extraction Kit (QIAGEN, Inc., Valencia, CA). Sequence analysis was performed on extracted and purified DNA by an automated DNA sequencer (Applied Biosystem Model 377, Foster City, CA) using the dideoxy terminator reaction method.

## RESULTS

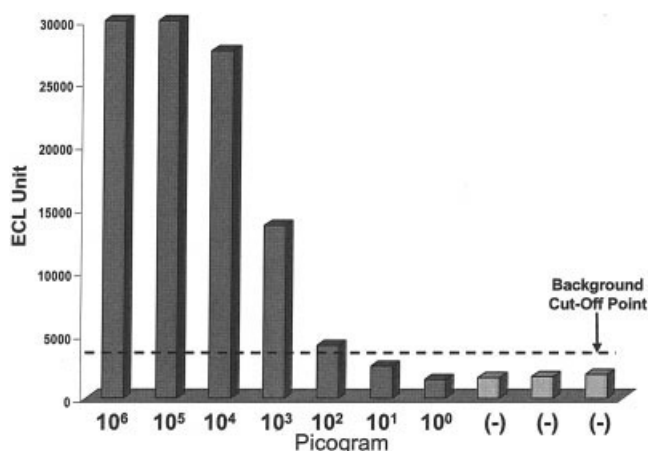
### Detection of K-ras mutations in cell lines

To assess the PNA clamp directed PCR assay's detection ability, we performed the assay with DNA from 4 established cell lines with known K-ras mutations and 5 established cell lines with no known mutations. PCR products were only detected in the 4 cell line samples containing the known K-ras mutations at codons 12 or 13 (Fig. 2). These PCR samples were analyzed using a semi-quantitative ECL ORIGEN® detection system. All 4 cell lines containing K-ras mutations produced very high ECL signals, whereas cell lines containing wtDNA produced minimal background signal. Four different K-ras point mutations in codon 12 and 13 were successfully detected in all K-ras mutant cell lines (Fig. 2).

Two different *in vitro* assays were carried out to assess the sensitivity and specificity of the K-ras detection system. Various dilutions of genomic DNA ( $1 \times 10^6$  to 1 pg) in molecular grade water from SW480 with K-ras mutation (GGT→GTT: codon 12) were prepared and assessed by PNA clamp PCR. The assay was sensitive at detecting K-ras mutation in as low as 100 pg of DNA without any carrier supplement (Fig. 3). For specificity, genomic DNA from SW480 cells was mixed in wtDNA from normal colon tissues in different ratios: 1:1, 1:10, 1:100, 1:500, 1:1,000 and 1:2,000. The PNA clamp PCR assay was carried out using 20 ng of DNA from the each of the mixtures preparation as template. K-ras mutation could be specifically detected at a dilution of 1:2,000 mutant to wild-type ratio (Fig. 4). These studies demonstrated the high specificity and sensitivity of the PNA clamp PCR method for detecting small amount of K-ras mutated DNA among a large pool of wtDNA. This approach provided an optimized and



**FIGURE 2** – ECL (bottom) and ethidium bromide stained gel (top) analysis of cell lines containing K-ras mutation (A–D), wtDNA (E–I) and blank control (J). Cell line allele mutation listed (A, GGT→GTT, codon 12; B, GGC→GAC, codon 13; C, GGT→TGT, codon 12 and D, GGT→TGT, codon 12).



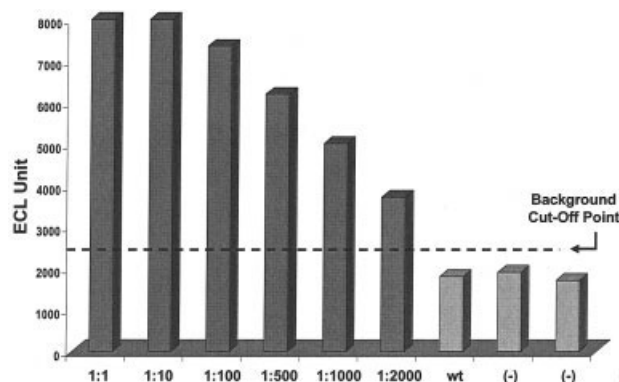
**FIGURE 3** – Serial DNA dilution assay. ECL detection of DNA extracted from SW480 cells containing K-ras mutation diluted in molecular biology grade water. Ten-fold dilutions of DNA from  $1 \times 10^6$  pg to 1 pg.

robust PCR-based assay for assessing primary CRC tumor cells for single-base K-ras mutations.

### Detection of K-ras mutation in primary tumors and paired SLN

The PNA clamp PCR assay was then optimized for the assessment of paraffin-embedded tissues. Initially, paraffin-embedded colon tumors, normal colon and noncancer lymph nodes were evaluated with this technique. All normal colon ( $n=10$ ) and noncancer lymph nodes ( $n=20$ ) were negative for K-ras mutation.

The histopathology of the SLN with H&E accurately reflected the pathologic status of the nodal basin in 67 (93%) of the 72 patients. Twenty-one (29%) of 72 patients' SLN were positive by H&E. IHC analysis of SLN using cytokeratin antibody staining identified metastases in an additional 16 of 46 patients, upstaging 35% of CRC. Furthermore, 4 of 5 patients with H&E negative SLN and positive non-SLN demonstrated occult tumor cells in the SLN by IHC. Seventy-two paraffin-embedded primary CRC specimens from patients who had undergone lymphatic mapping and SLN identification were assessed for the presence of K-ras mutations on codons 12 or 13 using the PNA clamp PCR technique (Fig. 5). Thirty (42%) of 72 patients' CRC primary tumors demonstrated K-ras mutations with this approach.



**FIGURE 4**—Dilutions of DNA from SW480 cells containing *K-ras* mutation in DNA from normal colon tissues. ECL detected *K-ras* mutant DNA with a sensitivity of 1:2,000, mutant to wtDNA.

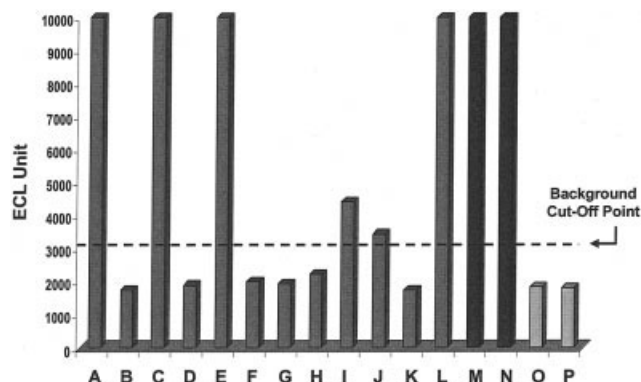
Among the 30 patients with *K-ras* mutations in their primary CRC tumors, 5 (17%) patients demonstrated metastases on H&E staining of their SLN and 7 patients (23%) demonstrated metastases on IHC analysis of their SLN. For the remainder 18 patients, their SLN had no evidence of metastases by both histopathology staining methods. PNA clamp PCR was performed on the SLN from all 30 patients with primary tumors demonstrating *K-ras* mutations. PNA clamp PCR analysis was positive in all 5 patients with H&E positive SLN and in 6 of 7 patients with IHC positive SLN. PNA clamp directed PCR detected more occult metastasis than standard H&E analysis of *K-ras* positive CRC, which was statistically significant (McNemar test,  $p = 0.014$ ). In 1 case, PCR did not detect *K-ras* tumor cells in an IHC positive SLN; subsequent to the negative PCR result, the SLN block was reassessed, and no tumor cells were detected on repeat IHC confirming accurate PCR analysis. The negative PCR and subsequent IHC results on the 1 case may be caused by the depletion of tumor cells in the tissue specimen from the previous serial sectioning for IHC investigation. In our study, correlation of *K-ras* expression to known histopathologic prognostic factors such as tumor size, grade, depth of invasion, lymph node involvement *etc.*, and patient outcome was assessed. Although significant correlations were not found, this pilot study demonstrated the accuracy and potential utility of the PNA clamp directed PCR in detecting metastases of *K-ras* mutation positive tumors.

#### *K-ras* sequencing

Sequence analysis was performed on 33 primary tumors found to be either *K-ras* positive ( $n=16$ ) or *K-ras* negative ( $n=17$ ) by PNA clamp PCR. In all instances, direct sequencing of the PCR product confirmed the presence or absence of the mutation as initially detected by the PNA clamp PCR. Four different patterns of *K-ras* mutation were detected at codon 12: GGT→GAT occurred in 6 patients; GGT→AGT in 2 patients; GGT→GTT in 5 patients and GGT→TGT in 1 patient. Tumors from 2 patients demonstrated the same mutation at codon 13: GGC→GAC. These results verified the accuracy of the PNA clamp PCR for detecting *K-ras* mutant tumor cells relative to the specific point mutation (Fig. 2).

#### DISCUSSION

Following the identification of multiple genetic alterations associated with the initiation and progression of CRC, molecular based assays have become the technique of choice for detecting these genetic events in occult tumor cells. Because mutation in *K-ras* is a relatively frequent occurrence in CRC (40–50%), develop early during tumorigenesis and remain consistently present during disease progression, this oncogene provides a unique ge-



**FIGURE 5**—Representative examples of *K-ras* mutation detection in CRC biopsies. Samples: A–L, colorectal cancer tumors; M and N, *K-ras* positive cell lines; and O and P, normal colon tissues. Positive sample cut-off point is 2 standard deviations above mean ECL U of the normal colon tissue controls.

netic marker for the molecular assessment of early stage metastasis to the regional lymph nodes.<sup>3</sup> In our study, we found 42% of primary CRC tumors contained *K-ras* mutation(s). *K-ras* mutation was detected by PNA clamp PCR in 6 of 25 patients with SLN considered tumor-free by H&E. There was only 1 case where PNA clamp PCR did not detect metastasis noted on IHC, and when we reassessed the SLN block by IHC, we identified no metastases suggesting that tumor cells may not have been in the sample evaluated by the PNA clamp PCR reaction.

In comparison, other studies that demonstrated upstaging of patients' lymph nodes with *K-ras* PCR did not perform IHC.<sup>27–29</sup> We would have upstaged 24% of the lymph nodes if IHC data was not considered (Table II). Furthermore, this technique would have accurately identified 4 of the 5 SLN patients considered false-negative using conventional H&E as an assessment tool. Currently, IHC is not routinely used to assess lymph nodes from CRC patients because it is impractical and costly. In combination with SLN mapping, IHC analysis would be a logistically feasible and cost-effective approach. However, the clinical implications of IHC detectable occult metastatic tumor cells in regional lymph nodes remains unclear.<sup>15,17,30</sup>

Thus, in our study we demonstrated a new, sensitive and accurate strategy in identifying micrometastases in paraffin-embedded SLN using a genetic marker. PNA clamp PCR for *K-ras* mutation was compared to the universally accepted technique of H&E for detection of SLN metastases and found to upstage a statistically significant greater number of patients. Although the combination of H&E and IHC improved the detection to equivalent level of PNA clamp directed PCR, the advantage of this innovative approach is that it is less labor intensive, highly accurate and not subjective to observer interpretation. Additionally, this assay can be performed on paraffin-embedded tissue sections allowing a practical application. Furthermore, the PNA clamp PCR can potentially be used to detect tumor progression in other cancers associated with specific single-base mutations. In contrast to cytokeratin antibody staining, which can be nonspecific for epithelial cells, molecular assays can accurately identify metastases in lymph nodes based on a consistent genetic aberration found in the corresponding primary tumor. This may also provide a method to validate metastatic tumor cells in the lymph node.

We assessed CRC patients using the highly comprehensive technique of lymphatic mapping, SLN identification, and IHC analysis of the SLN for identifying metastatic disease.<sup>31</sup> It has been shown that discordance can exist between *K-ras* mutations identified in the primary tumor and those found in paired bone marrow micrometastases from CRC patients.<sup>32</sup> These findings suggest that metastatic tumor cells may not always have a clonal relationship

TABLE II – SENTINEL LYMPH NODE STATUS FOR PATIENTS WITH K-RAS MUTATION PCR POSITIVE PRIMARY TUMOR

Sentinel lymph node status for patients with K-ras mutation PCR positive primary tumors (n = 30)				
5 (17%) H & E positive		25 (83%) H & E negative		
		7 (28%) IHC positive	18 (72%) IHC negative	
5 (100%) K-ras mutation PCR positive	0 (0%) K-ras mutation PCR negative	6 (86%) K-ras mutation PCR positive	1 (14%) K-ras mutation PCR negative	18 (100%) K-ras mutation PCR negative

with the primary tumor's genetic status or that the primary tumor can be of multifocal origin.<sup>33,34</sup> This finding may account for a false-negative result and must be considered in the development of any diagnostic assay. In addition, it is also suggested that tumors may acquire the K-ras mutation during progression;<sup>35</sup> therefore K-ras mutation may have potential use in identifying metastases even in patients whose primary tumors did not have the mutation.

This is the first report on the use of a genetic based assay to identify occult CRC metastases in SLN, which has been shown to be the first site of early stage metastases. Unlike RT-PCR methods of occult disease detection, genomic DNA templates are limited to 2 copies per cell, much fewer and therefore more difficult to detect.<sup>36</sup> However, DNA assays provide unique specificity that is difficult to achieve for RT-PCR markers.<sup>37</sup> Quantitative molecular assays also provide a facile tool that circumvents the subjectivity associated with morphologic methods that screen multiple sections for those rare tumor cells hidden among a background of other cell types. Because almost half of all CRC malignancies contain K-ras mutations, the presence of this alteration in primary tumors provides a uniquely specific target by which to search for occult

metastases. More recently, it has been suggested that the type of mutation on codon 12 (*i.e.*, glycine to valine) may be associated with more aggressive disease in patients with CRC.<sup>34,38</sup> Thus, in addition to the utility for assessing genetic events associated with cancer initiation and progression as a sensitive mutation detection tool, DNA based detection assays may have prognostic value when employed for the detection of occult metastasis. The clinical significance of these findings will require longer-term prospective follow-up, which is currently underway. Nonetheless, the hypothesis our pilot study evaluated is an important one, and a larger investigation would need to be undertaken to evaluate the clinical utility of detecting K-ras mutation using PNA clamp directed PCR. We have successfully demonstrated in our study the clinicopathological utility of SLN mapping combined with the PNA clamp PCR technique to efficiently identify micrometastases in the SLN of CRC patients. These findings provide the groundwork for a multiple gene-based detection assay which would improve occult tumor cell identification in a larger patient population. This approach may serve as a valuable complement to conventional histopathologic analysis for micrometastatic disease detection and patient prognosis.

## REFERENCES

1. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759–67.
2. Arends JW. Molecular interactions in the Vogelstein model of colorectal carcinoma. *J Pathol* 2000;190:412–6.
3. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988;319:525–32.
4. Losi L, Benhattar J, Costa J. Stability of K-ras mutations throughout the natural history of human colorectal cancer. *Eur J Cancer* 1992;28:1115–20.
5. Dix BR, Robbins PD, Spagnolo DV, Padovan GL, House AK, Iacopetta BJ. Clonal analysis of colorectal tumors using K-ras and p53 gene mutations as markers. *Diagn Mol Pathol* 1995;4:261–5.
6. Shibata D, Schaeffer J, Li ZH, Capella G, Perucho M. Genetic heterogeneity of the c-K-ras locus in colorectal adenomas but not in adenocarcinomas. *J Natl Cancer Inst* 1993;85:1058–63.
7. Sorenson GD. Detection of mutated KRAS2 sequences as tumor markers in plasma/serum of patients with gastrointestinal cancer. *Clin Cancer Res* 2000;6:2129–37.
8. Erlichman C, Fine S, Wong A, Elhakim T. A randomized trial of fluorouracil and folinic acid in patients with metastatic colorectal carcinoma. *J Clin Oncol* 1988;6:469–75.
9. Cohen AM, Tremittara S, Candela F, Thaler HT, Sigurdson ER. Prognosis of node-positive colon cancer. *Cancer* 1991;67:1859–61.
10. Jass JR, Miller K, Northover JM. Fat clearance method versus manual dissection of lymph nodes in specimens of rectal cancer. *Int J Colorectal Dis* 1986;1:155–6.
11. Cawthorn SJ, Gibbs NM, Marks CG. Clearance technique for the detection of lymph nodes in colorectal cancer. *Br J Surg* 1986;73:58–60.
12. Scott KW, Grace RH. Detection of lymph node metastases in colorectal carcinoma before and after fat clearance. *Br J Surg* 1989;76:1165–7.
13. Schmitz-Moormann P, Thomas C, Pohl C, Sohl R. Patho-anatomical demonstration of lymph node metastases in a surgical specimen. *Pathol Res Pract* 1982;174:403–11.
14. Wong JH, Severino R, Honnebieb MB, Tom P, Namiki TS. Number of nodes examined and staging accuracy in colorectal carcinoma. *J Clin Oncol* 1999;17:2896–900.
15. Cutait R, Alves VA, Lopes LC, Cutait DE, Borges JL, Singer J, da Silva JH, Goffi FS. Restaging of colorectal cancer based on the identification of lymph node micrometastases through immunoperoxidase staining of CEA and cytokeratins. *Dis Colon Rectum* 1991;34:917–20.
16. Adell G, Boeryd B, Franlund B, Sjdahl R, Hakansson L. Occurrence and prognostic importance of micrometastases in regional lymph nodes in Dukes' B colorectal carcinoma: an immunohistochemical study. *Eur J Surg* 1996;162:637–42.
17. Greenson JK, Isenhardt CE, Rice R, Mojzisek C, Houchens D, Martin EW, Jr. Identification of occult micrometastases in pericolic lymph nodes of Duke's B colorectal cancer patients using monoclonal antibodies against cytokeratin and CC49. Correlation with long-term survival. *Cancer* 1994;73:563–9.
18. Jeffers MD, O'Dowd GM, Mulcahy H, Stagg M, O'Donoghue DP, Toner M. The prognostic significance of immunohistochemically detected lymph node micrometastases in colorectal carcinoma. *J Pathol* 1994;172:183–7.
19. Morton DL, Wen DR, Wong JH, Economou JS, Cagle LA, Storm FK, Foshag LJ, Cochran AJ. Technical details of intraoperative lymphatic mapping for early stage melanoma. *Arch Surg* 1992;127:392–9.
20. Giuliano AE, Kirgan DM, Guenther JM, Morton DL. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. *Ann Surg* 1994;220:391–8.
21. Bilchik AJ, Giuliano A, Essner R, Bostick P, Kelemen P, Foshag LJ, Sostrin S, Turner RR, Morton DL. Universal application of intraoperative lymphatic mapping and sentinel lymphadenectomy in solid neoplasms. *Cancer J Sci Am* 1998;4:351–8.
22. Turner RR, Ollila DW, Krasne DL, Giuliano AE. Histopathologic validation of the sentinel lymph node hypothesis for breast carcinoma. *Ann Surg* 1997;226:271–6.
23. Ray A, Norden B. Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. *Faseb J* 2000;14:1041–60.
24. Uhlmann E. Peptide nucleic acids (PNA) and PNA-DNA chimeras: from high binding affinity towards biological function. *Biol Chem* 1998;379:1045–52.
25. Thiede C, Bayerdorffer E, Blasczyk R, Wittig B, Neubauer A. Simple and sensitive detection of mutations in the ras proto-oncogenes using PNA-mediated PCR clamping. *Nucleic Acids Res* 1996;24:983–4.
26. Taback B, Chan AD, Kuo CT, Bostick PJ, Wang HJ, Giuliano AE, Hoon DS. Detection of occult metastatic breast cancer cells in blood by a multimolecular marker assay: correlation with clinical stage of disease. *Cancer Res* 2001;61:8845–50.

27. Sanchez-Cespedes M, Esteller M, Hibi K, Cope FO, Westra WH, Piantadosi S, Herman JG, Jen J, Sidransky D. Molecular detection of neoplastic cells in lymph nodes of metastatic colorectal cancer patients predicts recurrence. *Clin Cancer Res* 1999;5:2450-4.
28. Hayashi N, Ito I, Yanagisawa A, Kato Y, Nakamori S, Imaoka S, Watanabe H, Ogawa M, Nakamura Y. Genetic diagnosis of lymph-node metastasis in colorectal cancer. *Lancet* 1995;345:1257-9.
29. Thebo JS, Senagore AJ, Reinhold DS, Stapleton SR. Molecular staging of colorectal cancer: K-ras mutation analysis of lymph nodes upstages Dukes B patients. *Dis Colon Rectum* 2000;43:155-9.
30. Noura S, Yamamoto H, Miyake Y, Kim B, Takayama O, Seshimo I, Ikenaga M, Ikeda M, Sekimoto M, Matsuura N, Monden M. Immunohistochemical assessment of localization and frequency of micrometastases in lymph nodes of colorectal cancer. *Clin Cancer Res* 2002;8:759-67.
31. Wood TF, Tsioulis GJ, Morton DL, Rangel D, Hutchinson W, Jr., Foshag LJ, Turner RR, Bilchik AJ. Focused examination of sentinel lymph nodes upstages early colorectal carcinoma. *Am Surg* 2000;66:998-1003.
32. Tortola S, Steinert R, Hantschick M, Peinado MA, Gastinger I, Stosiek P, Lippert H, Schlegel W, Reymond MA. Discordance between K-ras mutations in bone marrow micrometastases and the primary tumor in colorectal cancer. *J Clin Oncol* 2001;19:2837-43.
33. Giarretti W, Monaco R, Pujic N, Rapallo A, Nigro S, Geido E. Intratumor heterogeneity of K-ras2 mutations in colorectal adenocarcinomas: association with degree of DNA aneuploidy. *Am J Pathol* 1996;149:237-45.
34. Al-Mulla F, Going JJ, Sowden ET, Winter A, Pickford IR, Birnie GD. Heterogeneity of mutant versus wild-type Ki-ras in primary and metastatic colorectal carcinomas, and association of codon-12 valine with early mortality. *J Pathol* 1998;185:130-8.
35. Kinzler KW, Vogelstein B. Colorectal Tumors. In: Kinzler KW, Vogelstein B, eds. *The genetic basis of human cancer*. New York: McGraw-Hill, 1998.
36. Yun K, Merrie AE, Gunn J, Phillips LV, McCall JL. Keratin 20 is a specific marker of submicroscopic lymph node metastases in colorectal cancer: validation by K-RAS mutations. *J Pathol* 2000;191:21-6.
37. Bostick PJ, Chatterjee S, Chi DD, Huynh KT, Giuliano AE, Cote R, Hoon DS. Limitations of specific reverse-transcriptase polymerase chain reaction markers in the detection of metastases in the lymph nodes and blood of breast cancer patients. *J Clin Oncol* 1998;16:2632-40.
38. Andreyev HJ, Norman AR, Cunningham D, Oates J, Dix BR, Iacopetta BJ, Young J, Walsh T, Ward R, Hawkins N, Beranek M, Jandik P. Kirsten ras mutations in patients with colorectal cancer: the "RASCAL II" study. *Br J Cancer* 2001;85:692-6.