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Molecular Staging of Colorectal Cancer: K-ras Mutation Analysis of Lymph Nodes Upstages Dukes' B2 Patients

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MOLECULAR STAGING OF COLORECTAL CANCER: K-*RAS* MUTATION ANALYSIS OF LYMPH NODES UPSTAGES DUKES' B_2 PATIENTS

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MOLECULAR STAGING OF COLORECTAL CANCER: K-*RAS* MUTATION ANALYSIS OF LYMPH NODES UPSTAGES DUKES' B_2 PATIENTS

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The successful treatment of colorectal cancer depends upon the ability of staging systems to identify patients who are at risk for recurrence. The currently used systems fail in a significant number of patients. Multiple attempts have been made to improve upon these systems in order to provide better treatment for at-risk patients.

A variety of oncogenes and tumor suppressor genes have been implicated in colorectal cancer. Mutations in one of these, K-*ras*, occur in 40-60% of colorectal carcinomas. However, the relationship between specific mutations and clinical outcome is unclear.

The purpose of this study was to determine whether or not K-*ras* mutational status of tumors from 97 colorectal cancer patients was associated with survival, and to determine whether or not the K-*ras* mutational status of regional lymph nodes could be used as a staging tool.

The group was divided into four cohorts according to survival (less than five or greater than ten years) and modified Dukes' classification (B_2 and C_2). The author assessed techniques for DNA extraction and mutation detection. Tumor DNA was screened for K-*ras* mutations using mutation-specific DNA amplification, followed

by gel electrophoresis in a 96-well array. Dukes' B_2 patients with mutations were further analyzed to determine whether or not the same mutations could be identified in their lymph nodes.

Complete data was obtained for 89 patients (91.8%). Mutations were detected in 49.4% of tumors (44/89). Analysis of the mutations identified a group of 11 patients with concurrent mutations in codons 12 and 13, which was associated with long-term survival. Mutational analysis of lymph nodes from Dukes B_2 patients with mutation-positive tumors revealed an 80% (16/20) incidence of the same mutations in regional lymph nodes. None of the four patients with mutation-free lymph nodes developed recurrence compared to 37.5% (6/16) with K-*ras* positive lymph nodes.

The presence of concurrent mutations in codons 12 and 13 of the K-*ras* gene was a positive prognostic indicator in this group of patients. Mutational analysis of lymph nodes identifies high-risk patients who should be considered for additional treatment. Therefore, K-*ras* mutational analysis should be considered for molecular staging of colorectal cancer.

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CHAPTER I

COLORECTAL CANCER

Staging of Colorectal Cancer

With an age-standardized annual incidence rate for North America of 10-32 cases per 100,000 (1), colorectal cancer (CRC) is the second most common cause of cancer death (2). Treatment of CRC relies upon the identification of clinical and pathological features. These features have been used to develop staging systems, such as the Modified Dukes' system of CRC classification (3). Summarized in Table I, this system examines variations in the depth of tumor invasion, presence of lymph node metastasis, and presence of distant metastasis (4). However, this system fails to identify all at-risk patients. This is evidenced by lower than expected five-year survival rates following curative colorectal resections in patients with no identified metastasis (5).

The single most important prognostic factor affecting survival for patients undergoing curative resections for CRC is the presence or absence of regional lymph node involvement (6). It is also prognostically important to identify the number of regional lymph nodes containing metastatic deposits. This is evidenced by data generated by Cohen *et al.* (7), which show that 66% of patients with one to three positive nodes survived five years compared to 37% of patients with four or more

Table 1

Modified Dukes' Staging System

Dukes' Stage	Depth of Tumor Invasion	Lymph Node Metastasis	Distant Metastasis
A	Mucosa/Submucosa	No	No
B_1	Confined to bowel wall	No	No
B_2	Through bowel wall	No	No
C_1	Confined to bowel wall	Yes	No
C_2	Through bowel wall	Yes	No
D	Any depth	Yes/No	Yes

positive nodes. The difficulty in providing this information is two-fold. First, the mesentery must be appropriately evaluated so that a sufficient number of nodes can be retrieved and examined by the pathologist. Second, accurate identification of micrometastases, particularly in small nodes, remains a significant problem.

In addition to classical staging methods, it has been suggested that prognostic markers be used in choosing treatment for colorectal cancer (8). Several groups, including the American Joint Committee on Cancer (AJCC), have worked to develop recommendations for the use of prognostic markers.

The College of American Pathologists (CAP) recommended the routine clinical use of prognostic markers in addition to classical staging criteria (9). They divided prognostic markers into categories: (a) Category I—important prognostic

markers which are widely used in patient management, (b) Category II—markers which have been extensively studied and may be potentially useful, and (c) Category III—all others.

CAP Category I included histopathological markers such as venous invasion, carcinoma within adenoma, tumor type, and tumor grade. Serum carcinoembryonic antigen (CEA) was also included in this category. Category II included histological evidence of immune response and flow-cytometric analyses of DNA ploidy and S-phase fraction. Category III included flow cytometric analysis of mitotic index, serological markers other than CEA, cytokeratin staining, cytogenetic analyses, and genetic markers (9).

Adenoma-Carcinoma Sequence

The adenoma-carcinoma sequence has become a well-accepted model for the development of colorectal neoplasia (10). This model states that colorectal cancer develops through a multi-step process that develops from normal epithelium through hyperproliferative epithelium, adenoma, and carcinoma. The model is supported by visible changes in physical structure of the developing neoplasia (11). Additional support is found in the results of flow cytometric analysis of the DNA content of colorectal adenomas and focal cancers (12). These results indicate that DNA aneuploidy in adenomas correlates with the DNA aneuploidy of the associated focal cancers. However, adenomas not associated with focal cancers do not show DNA aneuploidy. Changes in DNA ploidy correlated with increased size of the developing

polyp, further supporting the idea of the adenoma-carcinoma sequence.

Genetics of Colorectal Cancer

The multi-step development of CRC is also supported by a variety of studies involving tumor suppressor genes and oncogenes. These studies suggest that 'multiple hits' are necessary for the development of CRC. Vogelstein *et al.* (13) suggested a model that involves mutation or loss of the *APC* (*a*denomatous *p*olyposis *c*oli) tumor suppressor gene, mutation of the K-*ras* gene, loss of the *DCC* (*d*eleted in *c*olon *c*ancer) tumor suppressor gene, and loss of p53 tumor suppressor function. Their model was supported by changes in mutation rates of these genes during tumor progression. In addition to these changes, other, yet to be identified, alterations may be responsible for metastatic potential of CRC (11).

CHAPTER II

THE K-RAS ONCOGENE

K-*ras* Structure and Function

The K-*ras* gene, located on chromosome 12, is part of a family of genes that encode 21 kDalton proteins (p21ras). The p21ras proteins are members of a superfamily of small GTPases that are involved in signal transduction and growth control (14).

The ras proteins have two domains. The first domain binds GTP or GDP and has GTPase activity (15). The protein cycles through an active GTP-bound form, and an inactive GDP bound form. This cycling is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (16). The second domain is at the C-terminus of the protein, which is responsible for translocation of ras to the plasma membrane (17). Ras proteins must undergo a series of post-translational modifications in order for this translocation to occur. Modification involves the addition of a farnesyl isoprenoid to the cysteine of the CAAX box, followed by removal of the AAX terminus and carboxymethylation of the terminal cysteine residue. The consensus C-terminal CAAX sequence is required for these modifications to occur (14,17).

Ras proteins are involved in multiple signal-transduction pathways. One

pathway involves mediation of signals from tyrosine kinase receptors following stimulation by growth factors (18). A complex of two proteins—SOS and Grb2, mediates ras activation following tyrosine kinase receptor stimulation. In its active GTP-bound form, ras is able to bind to and activate an effector, raf1 (19). Activation of raf1 triggers activation of the mitogen-activated protein kinase (MAPK) pathway. Ultimately, this results in activation of transcription factors and changes in gene expression (20).

Mutant forms of ras produce significant changes in cellular growth. This is attributed to either loss of GTPase activity or increased GDP/GTP exchange rates, which result in higher steady state levels of the GTP-bound form of the protein (21,22). The net result of the most common mutant forms of ras is decreased intrinsic hydrolysis rate of GTP by 10-fold (21).

The K-*ras* Gene

The K-*ras* gene (Figure 1) is over 38 kilo-base pairs long and consists of six exons—1a (also referred to as exon 0), 1, 2, 3, 4a, and 4b (23,24). The gene encodes two isomorphic ras proteins as a result of splicing of the alternate exons (4a or 4b).

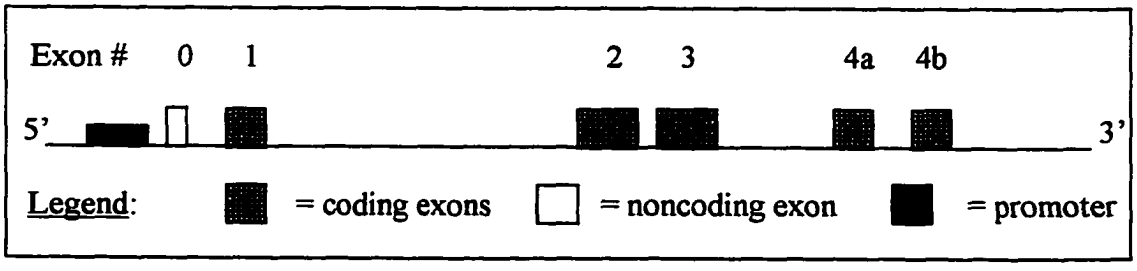


Figure 1. The K-*ras* Gene.

The two forms, 188 and 189 amino acids long, differ only in their carboxy terminus. The promoters lie immediately 5' to exon 1a, which is noncoding (25). K-*ras* oncogenic activity results from single point mutations at codons 12, 13 (exon 1) or 61 (exon 2), resulting in amino acid substitutions (26). Since these amino acids are located in the guanine nucleotide-binding domain of the ras proteins, this results in altered GTP binding or hydrolysis (17).

The K-*ras* Oncogene in Colorectal Cancer

The relationship between mutant forms of ras and oncogenesis is supported by a variety of evidence. Mutant forms of ras have been associated with a variety of human malignancies (26). Also, the functional activities of mutant ras are significantly different from normal ras protein, as evidenced by increased proliferation upon introduction of mutant ras into cells (27-29). Finally, transgenic mice with mutated ras exhibit a higher incidence of tumor development in the skin, liver, pancreas, and lungs (30).

K-*ras* mutations occur in 9-10% of small adenomatous colon polyps, 40-50% of larger dysplastic polyps, and 40-60% of colonic cancers (13,31-33). These findings suggest that, while K-*ras* mutation is not the initiating step in the adenoma-carcinoma sequence, it does occur fairly early in the progression (31,34,35).

Analysis of K-*ras* mutations has been used to determine the clonal nature of CRC. Dix *et al.* (36) examined 11 patients with colorectal tumors using K-*ras* and *p53* mutation analysis. Of the 11 patients, four had metastatic lesions of the lymph

nodes and/or liver. In all patients, they found complete homogeneity in respect to the mutations detected (i.e., the same mutation was found in adenoma, carcinoma, lymph nodes, and liver).

Others have performed similar analysis, but with slightly different outcomes. Giaretti *et al.* (37) found that, while the majority of tumors exhibited a homogeneous mutation pattern, in some tumors there appeared to be a subclone of cells that exhibited a different mutational picture. In two cases, a mutation was found in the deep tissue of the tumor, while there were no mutations found in the rest of the tumor. These subclones were also characterized by different DNA aneuploidy patterns. However, in the case of lymph node metastasis, the mutation pattern of the nodes matched that of the tumor.

Studying 40 early colonic carcinomas, Ohmura and Hattori (38) obtained similar results. While most cancers developed monoclonally, they found one case that demonstrated a mutation in the carcinoma that was not found in the adenoma. They also found a case that demonstrated one mutation in the adenoma and an additional mutation in the carcinoma. The authors suggested that these could have arisen from the "collision" of two clones. However, it may also be possible that the additional mutations occurred during tumor progression.

CHAPTER III

PURPOSE OF STUDY

At the time of publication of their recommendations for the use of prognostic markers in 1995, the College of American Pathologists suggested that K-*ras* would be the first genetic marker to be designated as a Category II prognostic marker (9). However, with conflicting views concerning the meaning of specific K-*ras* mutations, it would be difficult to use mutation information to influence treatment of colorectal cancer. It was the purpose of this study to further characterize the relationship between specific mutations and outcome in this disease.

Even if no relationship exists between the specific mutations found in tumors and the length of disease-free survival in colorectal cancer, it may be possible to use information concerning the mutational status of tumors, as a 'molecular finger print' to detect micrometastasis. Therefore, a further purpose was to determine whether or not micrometastasis, identified by the mutational status of lymph nodes as compared to associated tumors, was associated with the length of disease-free survival.

In order to accomplish the above, the author utilized paraffin-embedded archival material for which the necessary clinical and histopathological data was available. A variety of methods for extraction of DNA from this material and for K-*ras* mutation analysis were analyzed in order to develop methods that utilized this material optimally.

CHAPTER IV

ISOLATION OF DNA FROM ARCHIVAL MATERIAL

Background

Much of the early work to characterize the relationship between K-*ras* and colorectal cancer (CRC) was done with very small sample numbers of fresh tissue or cell lines. It is recognized that more extensive studies with long-term follow-up are needed to fully characterize this relationship. This would take many years to complete using fresh tissues. Consequently, it is very appealing to use archival tissue for which recurrence and survival data has been collected.

Many groups have attempted to use archival material, and there has been a virtual explosion of such work in the past five years. However, extraction of DNA suitable for analysis from these formalin-fixed, paraffin-embedded tissues has proven to be problematic. This can be evidenced by the variety of methods developed to improve upon previous methods, some of which work in as little as 20-30% of samples. A description of the available methods follows.

Methods Utilizing Microdissection for Enrichment

Some techniques rely upon microdissection of paraffin sections. The advantage of using microdissection is that the sample is enriched for the cells of

interest, i.e., tumor cells. This eliminates "background noise" from surrounding tissues, and thus increases the sensitivity of mutation detection methods.

Methods With Digestion and Purification

The earliest methods used for extraction of DNA from paraffin-embedded tissues followed the basic steps reported by Blin and Stafford (39) for the extraction of DNA from eukaryotic cells. These steps include cell lysis, protein digestion with proteinase K, protein precipitation, and purification of the DNA-containing supernatant with phenol and chloroform. This method was later modified by Sambrook, Fritsch, and Maniatis (40), and has become widely used for the extraction of DNA from mammalian cells.

Goelz, Hamilton, and Vogelstein (41) published a method in 1985 that included the steps of Blin and Stafford (39). Prior to the extraction, sections 6 μ thick were cut from paraffin blocks and stained with hematoxylin and eosin for histopathologic exam. These were used to guide the removal of at least 50 mg of tumor tissue from the intact paraffin block. Care was taken to trim away as much paraffin as possible, and the tissue was finely minced before proceeding. In their adaptation, lysis and digestion were performed twice—once for 24 hours and a second time for 40 hours, with vigorous vortexing prior to each incubation. Following digestion, the tissue was vortexed again, and forced through an 18-gauge needle three times. Purification of DNA was performed three times with phenol-chloroform (3 parts phenol, 4 parts chloroform, and 2 parts Tris-EDTA pH 9.0).

Goelz *et al.* (41) used their method to extract DNA from fresh frozen, fixed, fixed and embedded, and archival tissues. They examined the condition of the DNA following extraction and found that fixation in formaldehyde did not significantly affect results. However, the amount of DNA extracted from freshly embedded tissues was approximately half that of the tissues that were only fixed. Further, DNA extracted from archival tissue (embedded in paraffin and accumulated over a five-year period) was highly degraded. The size of recovered DNA ranged from 100 base pairs (bp) to 10,000 bp, with most of the DNA being between 100 and 1500 bp in length. They also determined that approximately 25% of the 30 samples tested were not suitable for use in blotting or cloning applications.

Others have since used the method of Goelz *et al.* (41) with slight variations. The most common variation is the use of microdissected tissue removed directly from the stained slide (42,43) or use of a stained slide to guide removal of tissue from an unstained slide (44,45). Still others have added a deparaffinization step, which uses xylene to remove the paraffin prior to digestion (46,47). These methods also decrease the digestion step to 24 hours (46) or to overnight (47-49). One group decreased digestion to overnight without deparaffinization (50).

Methods With Digestion and Without Purification

A further variation is to eliminate the DNA purification with phenol-chloroform following digestion. The digest is centrifuged, and the supernatant used in amplification protocols. This approach has been used to extract DNA from cells

scraped from either stained (51) or unstained slides (52-56). The method may also be preceded by deparaffinization with xylene (52-54).

Similar methods have been used, but with variations in microdissection techniques. One such variation is the use of ultraviolet (UV) light to destroy DNA from normal-appearing cells, thus isolating only DNA from tumor cells (57-59). With this technique a black marker is used to identify the location of tumor cells on the slide, and protect them from UV irradiation. After marking, the slide is placed marked-side down on a UV transilluminator for 2-4 hours. The tissue is then scraped from the slide into a microcentrifuge tube.

Being concerned by the possibility that marking in the above manner may not fully protect the tumor cells from irradiation, Turbett *et al.* (60) developed a technique to select cells of interest without scraping or irradiation. They used a nontoxic starch-based craft glue to identify the cells of interest. Xylene cyanole was added to the glue to aid in visualization. Areas of tumor cells were selected by covering them with the glue using a fine needle. Once the glue dried, the selections were lifted from the slide using forceps, and placed into lysis/digestion buffer. The authors found this method to be useful in the selection of areas varying greatly in size with ease.

Following extraction by the above methods, a portion of the digest was added to an amplification mix prior to cycling. A variation on this technique adds the amplification components directly to the full volume of digested sample (61). This eliminates further manipulations and minimizes opportunities for contamination.

Methods Using DNA Exposure

Still other methods eliminate both the DNA purification with phenol-chloroform and the protein digestion steps. These produce a crude extract that is used directly in amplification protocols. The idea behind this type of method is not only the elimination of time-intensive steps, but more importantly, the reduction of sample lost during multiple processing steps.

Finkelstein *et al.* (62) used a method in which tumor cells were selected by microdissection from deparaffinized slides and placed directly into an amplification mixture. They found efficiency of amplification of DNA extracted in this manner to be significantly reduced, and noted the presence of non-specific amplification products. Others have used similar techniques, but fail to comment on the level of success (63). Crude DNA exposure techniques have also been used without deparaffinizing the sample prior to microdissection (64-66)

Methods Without Enrichment

With some methods, DNA is extracted directly from intact paraffin sections without enrichment for tumor cells. These methods may or may not use xylene to wash away paraffin prior to extraction.

Methods With Digestion and Purification

These methods are similar to those of Goelz *et al.* (41), but with the elimination of microdissection. All groups who report using this method

deparaffinize with xylene prior to DNA extraction (13,44,67-71). After deparaffinization, the length of digestion varies from overnight up to 48 hours. Although the level of success in obtaining amplifiable DNA is rarely reported, one group reported obtaining DNA suitable for study in 45.3% (68/150) of the cases tested (71).

Methods With Digestion and Without Purification

As with techniques in which microdissection is used, some groups eliminate the phenol-chloroform steps of the protocol. This method is used with (13,33,72-77) or without deparaffinization (78,79) and with variations in length of digestion.

Wang *et al.* (80) evaluated three different methods for extraction from paraffin-embedded tissues, and found this method to be the best. They reported that neither the addition of deparaffinization with xylene nor purification with phenol-chloroform improved results.

Methods Using DNA Exposure

There are several variations of DNA exposure without microdissection. With the first method, paraffin sections are deparaffinized with xylene and mixing, the xylene is removed with ethanol washes, the sample is desiccated, and then boiled in either TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA pH 8.0) (78) or water (81-83) to release the DNA. A portion of this sample is added to the amplification mix before cycling. In another method, the step involving boiling in TE or water is eliminated.

Instead, the amplification mix is added directly to the desiccated sample, and the mix is subjected to boiling for 10 minutes prior to amplification (84,85).

Two groups have worked to improve upon this method by adding Triton X to the amplification mix to stabilize *Taq* DNA polymerase and aid in DNA release (86,87). With this method, a section from the paraffin block is placed directly into a reaction tube with the amplification mix, and heated at 95°C for 20 minutes prior to amplification. Although this reduces sample handling, it requires a large reaction volume in order to submerge the section.

Materials and Methods

PCR Design and Optimization

In order to evaluate methods for the extraction of DNA from paraffin-embedded archival tissue, the author first designed an amplification experiment. The design was then used to determine whether or not DNA extracted using a particular method would be suitable for mutation analysis.

Primer Design

After evaluating a number of either commercially available (Oncogene Point Primer Set, catalogue number APS07) or published primer sets (44,81,88,89) with variable results, the author designed primers using National Biosciences, Inc.'s OLIGO™ software package. A pair of primers was designed, which would produce a 275 base pair PCR product containing codons 12 and 13 of exon one. The primers,

which had essentially the same Tm and lacked significant hairpin or dimer formation, were manufactured commercially by National Biosciences, Inc. (NBI). These primers could also be paired with the Oncogene primers to produce 205 or 178 base pair products. The Oncogene primers amplify a 107 base pair region. Primer sequences were:

NBIa: 5' gtactggtggagtatttg 3' NBIb: 5' cagagaaacctttatctg 3'

OncA: 5' gactgaatataaacttgtgg 3' OncB: 5' ctattgttggatcatattcg 3'

PCR Optimization

In order to produce the most product with the highest level of specificity (i.e., no nonspecific product), the PCR conditions must be optimal. The annealing temperature was first determined, and then the concentration of four reaction components ($MgCl_2$, primers, dNTPs, and target DNA) were optimized at that temperature.

Annealing temperature is critical to the achievement of optimal PCR results. Poor yield of product will result from an annealing temperature that is too high, while a low annealing temperature will result in non-specific PCR products (90). The annealing temperature for this experiment was determined using National Biosciences, Inc.'s OLIGO™ software package.

Optimizing amplification conditions can be a time-consuming proposition. For example, if one were to test four different reaction components at three different concentrations, it would take 81 reactions to find the optimal combination! Cobb *et*

al. (91) reported using Taguchi's engineering method to optimize PCR. The method was developed by Taguchi to optimize industrial design processes using a minimal number of experiments.

The Taguchi method utilizes the properties of an orthogonal array to determine the effect of one component on another. Using Cobb's (91) adaptation of this method, four PCR components ($MgCl_2$, primers, dNTPs, and target DNA) at three concentrations were evaluated in nine reactions. Table 2 shows the components and concentrations used to determine the optimal conditions for initial amplifications.

The concentrations to be used were determined by first estimating level B for

Table 2

Components and Concentrations Applied to Taguchi Array

		Concentrations			
Component		A	B	C	Units
I	Primers	0.4	0.8	1.8	μM
II	Template	0.2	1.0	2.0	μg/100μl
III	$MgCl_2$	0.5	1.5	4.0	mM
IV	dNTP Mix	60	100	200	μM

each component. This was done by a review of reported concentrations used in amplification of this gene, and the manufacturer's recommendations (Perkin Elmer GeneAmp Core Reagents Kit). Levels A and C were then determined by using the most extreme conditions reported for this gene.

The PCR components were distributed into nine tubes, following the Taguchi array shown in Table 3. Note that, between any two columns, all combinations of concentrations (designated by A, B, and C) have the same frequency.

Table 3

3 X 4 Taguchi Array

	Component			
Reaction #	I	II	III	IV
1	A	A	A	A
2	A	B	B	B
3	A	C	C	C
4	B	A	B	C
5	B	B	C	A
6	B	C	A	B
7	C	A	C	B
8	C	B	A	C
9	C	C	B	A

DNA for this optimization procedure was obtained from fresh tumor cells derived from the pleural fluid of a patient with lung cancer, which was donated by the West Michigan Flow Cytometry Laboratory in Grand Rapids, Michigan. Genomic DNA was extracted from this sample using the Puregene DNA Isolation Kit, manufactured by Gentra Systems, according to instructions provided with the kit. Briefly, the cells were washed with Cell Suspension Solution and pelleted by centrifuging at 1000 X g for 15 minutes in a refrigerated centrifuge. The pellet was resuspended in Cell Lysis Solution and proteinase K was added to a concentration of 100 μg/ml. The mixture was incubated for two hours at 55°C. The protein was precipitated with the Protein Precipitating solution provided, and pelleted by centrifuging at 16,000 X g for 3 minutes. DNA was precipitated from the supernatant using isopropanol, and pelleted by centrifuging at 16,000 X g for 5 minutes. After washing the pellet once with 70% ethanol, and allowing it to dry at room temperature, the DNA was resuspended in DNA Hydration Solution by heating at 65°C until dissolved, and stored at –70°C until use.

Amplification of a 275 bp region of exon 1 (NBI primers described above) was performed in a 100 μl total reaction volume using the Perkin Elmer Core PCR kit and run on a Perkin Elmer 480 thermocycler (additional reaction conditions: 10 mM Tris-HCl pH 8.3, 50 mM KCl, and 2.5 Units *Taq* polymerase). The tubes were gently mixed and overlaid with mineral oil prior to amplification. The amplification program consisted of an initial denaturing step for 5 minutes at 95°C, 30 cycles of PCR, and a final extension of 10 minutes at 72°C. Each PCR cycle consisted of 1

minute at 95°C, 2 minutes at 52°C, and 1 minute at 72°C.

Following amplification, the PCR products were electrophoresed at 80 Volts for 40 minutes in a 2% GTG agarose gel (Seakem) containing ethidium bromide (Figure 2). The gel was photographed with a Polaroid camera using ultraviolet epi-

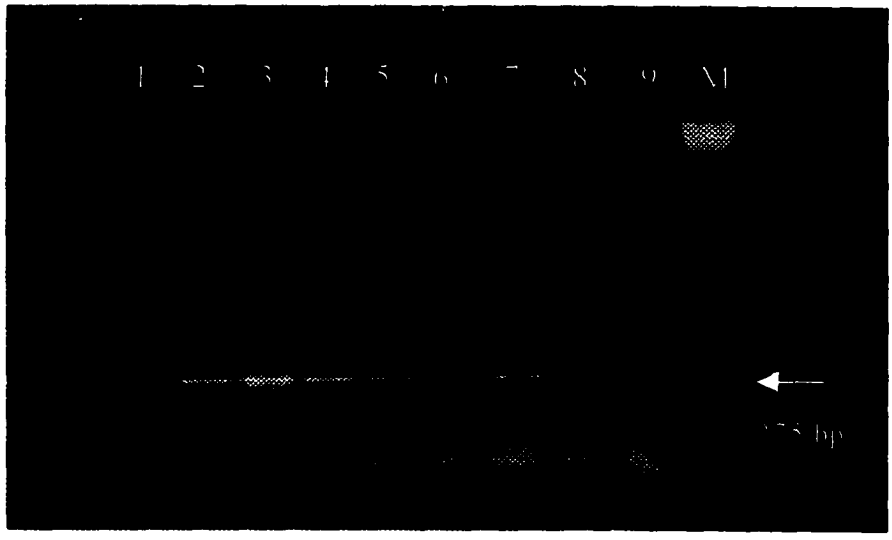


Figure 2. PCR Optimization Gel.

illumination, and the yield of PCR product for each reaction was visually scored. The quadratic loss function was applied to determine signal-to-noise ratios (SNL) for each concentration:

$$SNL = -10 \log \left[\frac{1}{n} \sum_{i=1}^{n} \frac{1}{y_i^2} \right]$$

Where: n = number of levels, y = yield, and i = index.

For any component, the concentration with the highest signal-to-noise ratio was designated as the optimal of the concentrations tested. This calculation was

repeated for each of the components tested. The concentrations determined to be optimal for the four components were combined for future amplifications (4.0 mM $MgCl_2$, 200 μM each dNTP, 0.8 μM each primer, and 1.0 μg target DNA). Note that this combination was not represented in the nine reactions performed here. These conditions were subsequently used to evaluate extraction methods.

Anticipating that this method might be used multiple times, this author developed a simple computer program for doing this calculation and reporting the optimal level for each component. The program, which may be found in Appendix A, runs in Microsoft Q Basic. This modification of Taguchi's method was used to optimize all amplification reactions used in this work.

Methods for DNA Extraction From Archival Material

A variety of extraction methods were used by this author to determine the optimal approach for the extraction of amplifiable DNA from archival tissue. The success of each method was analyzed by amplifying a 275 bp region of the K-*ras* gene from each DNA sample using the PCR program described above with conditions determined through the described optimization (final conditions: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 4.0 mM $MgCl_2$, 200 μM each dNTP, 0.8 μM each NBI primer, 1.0 μg target DNA, and 2.5 Units *Taq* polymerase in a total reaction volume of 100 μl). A summary of the methods used is shown in Table 4. These methods are listed in order of complexity, not in the order of trials. Unless indicated, the exact conditions for each step followed a standard protocol, described below.

Table 4

Methods Used for the Extraction of DNA From Archival Tissues

Method #	Reference #/ Or Kit Used	Sections Used	Xylene	Cell Lysis/ Digestion	DNA Extraction	AN[1]	A%[2]
1	(40)	5-10 x 10 μ	1-3 washes	8-72 hours 55°C	Organic	1/10	10
2	(92)	5-10 x 10 μ	1-3 washes	2 hours 55°C [a]	Organic [a]	0/30	0
3	(33,52)	5-10 x 10 μ	1-3 washes	14-16 hours 55°C	None	1/5	20
4	Puregene	5 x 10 μ	1 wash	Per kit	Per kit	1/3	33
5	Puregene	5 x 10 μ	1 wash	Per kit	Protein Precipitated [b]	1/3	33
6	Puregene	5 x 10 μ	1 wash	Per kit	None	0/3	0
7	Puregene	5 x 10 μ	1 wash	None	None	0/3	0
8	(60)	1 x 10 μ	None	16-24 hours 55°C	None	4/13	31

Table 4 –Continued

Method #	Reference #/ Or Kit Used	Sections Used	Xylene	Cell Lysis/ Digestion	DNA Extraction	AN[1]	A%[2]
9	(93)	1 x 10 μ	None	20 minutes in ROSE [c] with mixing at 90°C	None	13/62	21
10	GeneReleaser	1 x 10 μ	None	1-2 minutes in reagent microwaved on high	None	0/5	0
11	(94)	1 x 10 μ	None	1-2 minutes in TE microwaved on high	None	0/10	0
12	(65)	1 x 10 μ	None	Boiled in 100 μl TE For 10 minutes	None	0/10	0
13	(81)	1 x 10 μ	2 washes	Boiled in 100 μl sterile water for 10 minutes	None	2/3	67
14	None	1 –5 x 10 μ	None	Boiled in water for 15 minutes, 100 μl/ section	None	59/71	83
15	None	1 –5 x 10 μ	None	Heated in Thermomixer 15 minutes at 95°C	None	5/10	50

Table 4 –Continued

Method #	Reference #/ Or Kit Used	Sections Used	Xylene	Cell Lysis/ Digestion	DNA Extraction	AN[1]	A%[2]
16	None	1 –5 x 10 μ	None	Heated in Thermomixer 30 minutes at 95°C	None	3/10	30
17	(55)	1 x 10 μ	None	None [d]	None	0/5	0

Legend: 1 = AN represents the number of successful amplifications/number of attempts

2 = A% represents the % of successful amplifications

a = Digestion and extraction performed in a Serum Separation Tube (SST) to reduce sample transfer

b = Digest used directly for PCR after protein is precipitated

c = ROSE (Rapid One-Step Extraction) buffer, 10 mM Tris-HCl pH8.0, 312.5 mM EDTA pH 8.0, 1% sodium lauryl sarkosyl, and 1% polyvinylpolypyrrolidone (PVPP)

d = Paraffin section placed in amplification mix and heated at 95°C for 10 minutes prior to amplification

Deparaffinization washes were performed with 1.0 ml of xylene, incubated for 5 minutes, and aspirated following centrifugation at 16,000 X g for 5-10 minutes. Following the number of washes indicated, the xylene was washed away with 0.5 ml of ethanol, and the pellet dried at 37°C.

If a kit was used, 600 µl of the lysis solution provided with the kit was added to the cell pellet, along with proteinase K to a concentration of 100 µg/ml, and the sample was incubated as shown. If a kit was not used, cell lysis and digestion was performed in 1 ml of digestion/lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0, 100 mM NaCl, 3.0% SDS, 100 µg/ml proteinase K), and the sample was incubated as shown.

For organic methods, three extractions were performed with phenol-chloroform-isoamyl alcohol (25:24:1). Each extraction was performed in 1ml, centrifuged for 5 minutes at 16,000 X g, with the aqueous layer used for the next step. Following the third extraction, the DNA was precipitated from the aqueous layer with 1.0 ml of ice cold absolute ethanol, pelleted by centrifuging at 16,000 X g for 15 minutes, rinsed once in 1 ml of 70% ethanol, dried in a centrifugal vacuum concentrator, and resuspended in TE.

For nonorganic extractions, protein was precipitated from the digest using a protein precipitating solution (provided in the kit), and centrifuged for two minutes at 16,000 X g. DNA was precipitated from the supernatant as described for organic methods.

Initially, methods were tried repeatedly (methods 1, 2, 8, and 9), then trials

were limited to 10 (methods 1, 11, or 12). In later trials, only methods that worked for the first 3-5 samples were further analyzed. Methods 13 and 14 were evaluated simultaneously. Although method 13 was successful in 2 of the first 3 reactions, method 14 produced amplifiable DNA in all initial tests. Since method 14 was initially successful and eliminated the use of xylene, it was further evaluated.

Using method number 14, which consisted of boiling one 10 micron thick section in 100 µl of sterile water for 15 minutes, experiments were designed to determine whether the method could be improved. Parameters investigated were: (a) the thickness of the sections, (b) the number of sections per volume of water, (c) the method of heating, (d) effect of mixing during heating, (e) the effect of centrifuging following heating, (f) the effect of cooling following heating, (g) the effect of storage, and (h) the effect of using a set volume of extract in amplification experiments.

For evaluation of section thickness, sections were cut that were 5 microns, 10 microns, and 20 microns thick and boiled in 100 µl sterile water. It was determined that amplification was optimal with sections 10 microns thick. The author theorized that 5 microns did not provide enough tissue, and that excess paraffin may have interfered when using sections 20 microns thick.

The effect of the number of sections used was tested by using 1, 5, or 10 sections (10 microns thick) in 100-500 µl of sterile water. Ten sections were found to be difficult to work with. One section in 100 µl of sterile water produced the optimal PCR product, and was also easiest to handle.

Two methods of heating were evaluated: (1) boiling and (2) heating at 95°C

in an Eppendorf Thermomixer, with no difference detected. The effect of mixing while heating was evaluated in the Thermomixer, set to the lowest speed. It was theorized that mixing may aid in disbursement of paraffin and cells, but no difference was found.

Since condensation formed on the inside of the tubes, the author thought that centrifugation should be performed to avoid aerosols, which may result in carryover. Samples were briefly centrifuged (10-15 seconds at 16,000 X g) to collect the samples into the bottoms of the tubes. Since no difference in amplification was found by adding this step, and it was a good preventative measure, it was added to the protocol.

Following heating, samples were cooled at 4°C or on ice for 5-15 minutes before testing. This improved results slightly, with no effect after 10 minutes. There was no difference observed between the two temperatures, except that the paraffin hardened more on ice. Since the paraffin formed a barrier on top of the sample, this made removal of the sample more difficult. Therefore, cooling for 10 minutes at 4°C was used.

The effect of storage was evaluated at room temperature, 4°C, -20°C, and –70°C. At room temperature, the samples were unamplifiable after a few hours. When stored at 4°C, the samples amplified after 2-3 days, while this was extended to approximately 2 weeks when stored at –20°C. Long-term storage was only possible when the samples were placed at –70°C.

Although the optimal concentration for target DNA was determined by the

Taguchi method to be 1 μg/100 μl using fresh tissues, there seemed to be some variation in results from paraffin block to paraffin block, which appeared to be associated with the volume of extract used in amplification. Noting that some other authors used a set volume of extract rather than a set amount of DNA in amplification reactions, the author decided to evaluate this approach.

Experiments were designed to evaluate the addition of 10, 25, or 40 μl of crude extract to a 100 μl total reaction volume. Amplification was most consistent using 25 μl of DNA extract, regardless of DNA concentration. This may have been caused by the presence of PCR inhibitors in the paraffin blocks, which could have been released during extraction. This theory was tested by adding varying amounts of the paraffin to an extract of fresh cells and heating, and was found to be correct--PCR was inhibited by this addition. This would also explain why using thicker or a greater number of sections had a negative effect in previous experiments.

While performing these tests, it was noted that processing the samples in large batches yielded poor results. It was subsequently determined that small batches of 8 to 10 samples worked best, and extractions were performed in this manner in all subsequent experiments.

Results

As a result of these tests, a standard method was developed for the extraction of DNA from paraffin blocks. A single 10 micron-thick section was removed from each paraffin block using a disposable microtome blade, and placed into a sterile

microcentrifuge tube. All instruments were thoroughly cleaned with chlorine bleach followed by ethanol, and gloves were changed between each sample to prevent cross-contamination. For extraction, no more than 8-10 samples were processed simultaneously. Sterile, ultrafiltered, deionized water was added to each tube in the volume of 100 µl. The tubes were sealed and heated at 95°C for 15 minutes, and briefly centrifuged to collect the sample into the bottom of the tubes, followed by cooling for 10 minutes at 4°C. The layer of paraffin was pierced with a pipette tip, and the supernatant was transferred to a second sterile tube and stored at -70°C until use. Twenty-five µl of each extract was used in a 100 µl amplification reaction. This method will be referred to as 'modified method 14'.

Discussion

The DNA from paraffin-embedded archival material is generally of poor quality (41) due to damage during the formalin fixation process (81). This makes isolation of DNA suitable for analysis a challenge. A variety of methods for the extraction of DNA from archival material were reviewed by this author. Many of the methods enriched for tumor cells by using microdissection. This required that multiple slides be prepared from each tissue block to be tested. The current project examined tumors from approximately 100 patients and an additional five to ten blocks of lymph nodes from 20 patients. This would require that approximately 2000-3000 slides be prepared, which was not within the scope of the project. Furthermore, lymph node micrometastases in Dukes' B_2 patients could not be

visualized with histopathological analysis, making enrichment for tumor cells in lymph nodes impossible with this method. Therefore, an approach that did not require microdissection was sought.

There was a risk that the small amounts of DNA that would be suitable for amplification may have been lost during the extraction process. This risk could be minimized with the elimination of steps from the extraction method. Several groups have tried this approach. After evaluating the existing methods, this author theorized that paraffin might be satisfactorily removed while performing heat lysis of the cells, eliminating the need for deparaffinization with xylene. Since a fairly high volume of extract was transferred to the amplification reaction, the composition of the lysis solution was critical. Ions and chemicals contained in the solution could affect the processivity of the polymerase. Therefore, water was used as the lysis solution, to eliminate the transfer of unwanted additional components to the amplification mix.

Even though this method yielded a crude DNA extract, it proved to be very useful for this project. Additionally, since there were few manipulations of the samples, the risk of cross-contamination events was minimized.

Conclusions

Of the methods analyzed, the simplest proved to be the most successful and reliable (modified method 14, page 29). Amplifiable DNA was obtained from a single paraffin section by heating in sterile water. Manipulations of the sample were minimized, reducing the possibility of cross-contamination or loss of valuable DNA.

CHAPTER V

MUTATION DETECTION AND ANALYSIS

Background

The incidence of mutations, as well as the exact nature of mutations in respect to location and type, varies considerably depending upon the selection of samples and the analytical method used. There are a variety of methods used to detect mutations in the K-*ras* gene. These methods vary in sensitivity and specificity. This section will give an overview of these methods.

DNA Sequencing

PCR followed by direct sequencing has been used to detect K-*ras* mutations in lesions of the mouse liver (81), as well as human tumors located in the pancreas (44), esophagus (45), and colon (62,63,71,73,95). Although sequencing is generally regarded as the 'gold standard' when it comes to identification of specific mutations, it is only able to detect mutant sequences that represent approximately 25% or more of the total sample (62,96). For this reason, microdissection is generally used to enrich for cells of interest prior to DNA extraction and PCR amplification.

Single-Strand Conformation Polymorphism

PCR followed by single-strand conformation polymorphism (SSCP) is a mutation detection method, which relies upon the folding of single-stranded DNA, and assumes that a point mutation will result in a conformation change that will alter mobility during electrophoresis. Reliability of SSCP is highly dependent upon electrophoretic conditions, which may also affect mobility. Since the electrophoresis may continue for up to 18 hours (72), a system for controlling the temperature of the gel is employed (35).

This method has been used to detect mutations in gastric tumors (97), pancreatic tumors (98), lung tumors (88), bone lesions (80), and breast tumors (88), as well as in tumors of the colon and rectum (36,52,72) SSCP is reported to detect as few as 10% mutant sequences in a background of normal DNA (36).

SSCP is limited to the detection of mutations, and is unable to identify the specific type or location of point mutations, even when the mutation is known prior to analysis (52). Therefore, positive results are often confirmed with sequencing in order to identify the specific mutation present (36,52,72,88). However, without enrichment prior to sequencing, 23% of mutations detected with SSCP are not confirmed (72). Therefore, it is advisable to use microdissection to enrich for tumor cells.

Dot Blot Analysis

Point mutations can alter the ability of DNA probes to bind to specific DNA

sequences. Dot blot analysis takes advantage of this specificity using oligonucleotide probes under stringent conditions. DNA is blotted onto a membrane, such as nylon, and then hybridized to mutation-specific oligonucleotide probes.

Dot blot analysis has been used to identify K-*ras* mutations in tumors of the ovary (96), lung (58,75) pancreas (66), and colon (13,33,35,38,83,99,100). Results vary according to the probes used, hybridization conditions, and stringency of washes. Under proper conditions, only perfectly matched probes should hybridize to the DNA target sequence. When this is achieved, single-base discrimination is possible (100). However, a separate probe must be made to detect each possible mutation. This would require 16 different probes for each codon, and would still not allow the detection of concurrent mutations in both codon 12 and 13 (99).

Mutant-Enriched Restriction Fragment Length Polymorphism

A variation of the restriction fragment length polymorphism (RFLP) has been designed to create artificial restriction sites in an area of interest. This method, designated here as mutant-enriched RFLP (ME-RFLP), has also been referred to as mismatched-primer mediated PCR-RFLP (101), more simply as mismatched PCR-RFLP (102), primer-mediated RFLP (103), or as amplification-created restriction sites (ACRS) (53).

Applied to mutation detection in K-*ras*, the simplest of these methods uses a mismatched 3' PCR primer to create a *Bst* NI restriction site that extends into codon 12 (34,76,77,104) (Figure 3). Following PCR amplification, products are digested

with *Bst* NI. Only DNA containing the wild-type sequence in codon 12 is successfully digested, as any mutation in the first two positions of codon 12 would eliminate the restriction site. The digest is then analyzed using polyacrylamide gel electrophoresis (PAGE) (7,76) or high-resolution agarose electrophoresis (105). Following electrophoresis, the gel is stained with ethidium bromide for visualization (106).

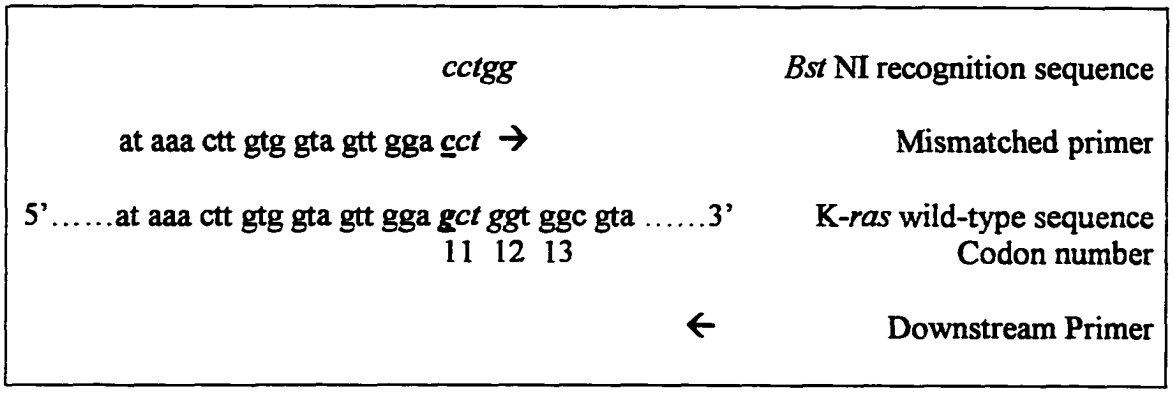


Figure 3. Primer Design for ME-RFLP.

The most common variation of ME-RFLP is the two-stage, or mutant-enriched method (105). With this method, following PCR and restriction digest, a second round of PCR is performed (70). This may be followed with a second digest (42,43,79,103) One group has added a third-stage of PCR and digestion (107).

Another variation is separate testing for mutations at codon 13 with the creation of a different restriction site, most commonly *Hph* I (108), which only detects GGC → GAC mutations (109). Creation of other restriction sites at codons 12, 13, and 61 have also been used (54,106,107,101,110). The length of digestion varies considerably, with digestion for as long as 16 hours (105).

ME-RPLP has been used to screen for mutations in tumors of the lung (54,102), stomach or esophagus (56,108), pancreas (76,79), and endometrium (53), as well as in the colon and rectum (42,43,51,57,70,101,107-112). While the method identifies the presence of a point mutation in position 1 or 2 of codon 12, it does not identify the specific type of mutation.

Allele-Specific Polymerase Chain Reaction

Allele-specific polymerase chain reaction (AS-PCR) is a variation of PCR, which relies upon a requirement of *Taq* polymerase that there be a perfect match between the 3' end of the DNA primer and its target sequence (89). Since *Taq* polymerase lacks proof-reading ability, a mismatch at the extreme 3' end of the primer will prevent the polymerase from extending the DNA strand (67,79). This method can be used both to detect and to identify specific mutations, eliminating the need for sequencing. It has also been referred to as mutant allele specific amplification (MASA) (113).

AS-PCR is useful for detecting and characterizing mutations in genes (such as K-*ras*) that have specific sites where mutations have been identified. By positioning the 3' end of the primer directly over the nucleotide in question, and varying the 3' nucleotide of the primer, the nucleotide on the target DNA can be identified (89) (Figure 4). Following amplification, products are subjected to agarose gel electrophoresis and visualized with ethidium bromide staining.

AS-PCR in its simplest form has been used to detect a single, specific

mutation found in the blood cells of patients with acute myelogenous leukemia (114). With this application, a single mutant-specific primer was used to screen for malignant cells.

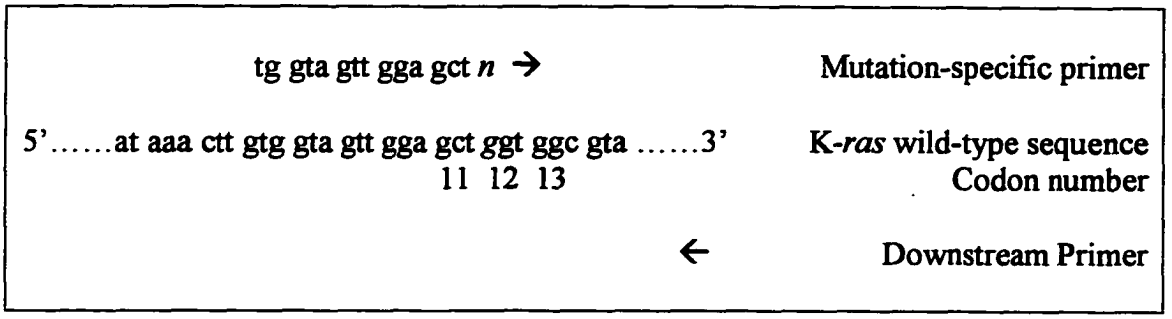


Figure 4. AS-PCR Primer Design for Position 1 of Codon 12.

When applying this method to colon cancer, most groups have screened for all possible mutations of codon 12. Some groups have added screening for the most common glycine to aspartic acid mutation of codon 13 (115), while others have also screened for serine or arginine at this site (65,78). A more aggressive approach has been used to screen for all possible mutation at all three of the above mentioned positions, requiring twelve mutant-specific primers (113) (Figure 5).

A further variation of this method is to employ multiplex PCR to screen for all mutations of a particular position at the same time, in order to reduce the number of amplification reactions required (67,78,113). With this approach, only the position of the mutation can be determined in the original screening reactions. Additional tests must be run to determine the exact type of mutation in a positive screen.

In studying mutations in liver cancer, Stork *et al.* (89) have applied this method to the detection of K-*ras* mutations in the first two positions of codon 12

With their approach, position one is screened using a mutant-specific primer in the sense direction, while a mutant-specific primer in the antisense direction is applied to position two (Figure 6). When designed in this fashion, mutations in position one do

tg gta gtt gga gct ggt *gn* →	13b mutation-specific primer
tg gta gtt gga gct *gn* →	12b mutation-specific primer
tg gta gtt gga gct *n* →	12a mutation-specific primer
5'..... ctt gtg gta gtt gga gct ggt ggc gta ggc aag .....3'	K-*ras* wild-type sequence
12 13	Codon number
←	Downstream Primer

Figure 5. AS-PCR Primer Design for Codon 12 and Position 1 of Codon 13.

tg gta gtt gga gct *n* →	12a mutation-specific primer
5'..... ctt gtg gta gtt gga gct ggt ggc gta ggc aag .....3'	K-*ras* wild-type sequence
11 12 13	Codon number
←	Downstream Primer
→	Upstream Primer
5'.....gta gtt gga gct ggt ggc gta ggc aag agt gcc.....3'	K-*ras* wild-type sequence
11 12 13	Codon number
← *n*a ccg cat ccg ttc tca	12b mutation-specific primer

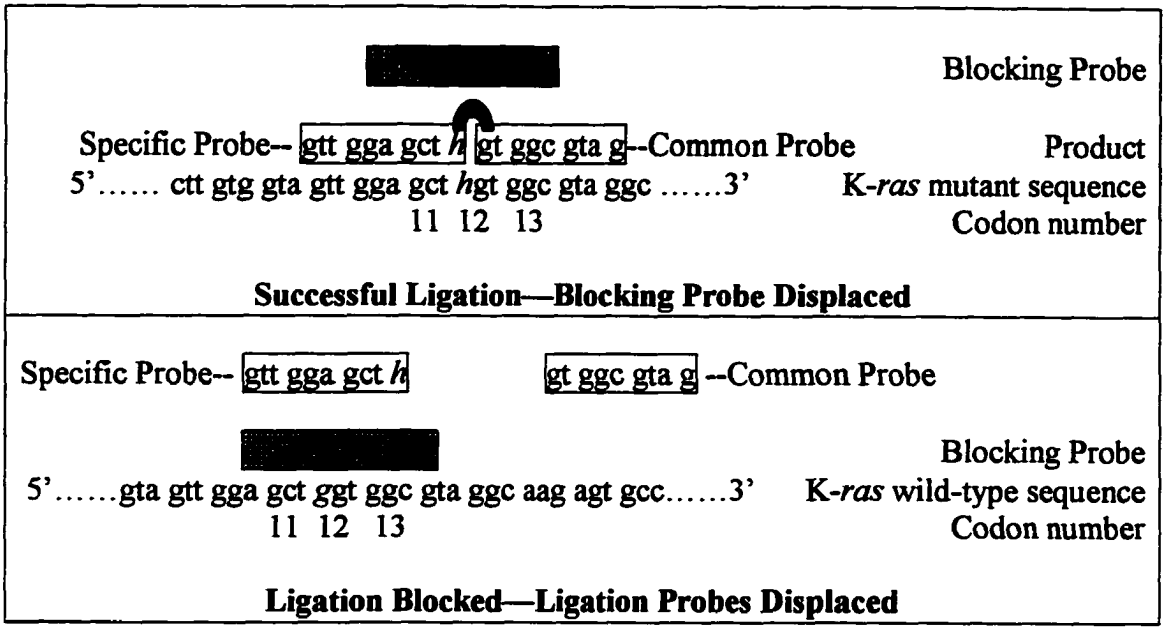
Figure 6. Opposing AS-PCR Primer Design for Codon 12.

not interfere with detection of mutations in position two. However, codon 13 mutations can not be analyzed with this method.

Allele-Specific Ligation Assay

A variation of the ligation assay, the allele-specific ligation assay combines the most attractive features of ligation and AS-PCR. In the ligation assay, two labeled probes are designed to anneal side-by-side on the target DNA. Following annealing, a ligation reaction joins the two probes into a single, longer sequence. If both probes anneal in the proper orientation, ligation will occur and the larger ligation product will be seen when analyzed electrophoretically, otherwise bands corresponding to the two smaller probes will be seen.

For the allele-specific ligation assay, the mutant-specific probes are designed similarly to the mutant specific primers of AS-PCR. The second, or common, probe is designed just 3' of the mutant-specific probe (116). A third probe (the blocking probe) overlaps the site of ligation (117) (Figure 7). The blocking probe is perfectly matched to the wild-type sequence, and competes with the mutant-specific probe. Since oligonucleotides with a single base mismatched bind weaker than perfectly matched oligonucleotides (84), this decreases the opportunity for non-specific ligation. This assay has been used to detect K-*ras* mutations in colorectal cancer (117,118). The method does not detect multiple mutations unless probes are designed specifically for their detection (116).



Legend: *h* = a, t, or c = ligase

Figure 7. Allele-Specific Ligation Assay for Position 1 of Codon 12.

RNAse Mismatch Cleavage Assay

When RNA extraction is possible, the RNAse mismatch cleavage assay may be used for mutation detection. Specific, labeled RNA probes are annealed to the region of interest. If mismatches are present, single-stranded regions are available for RNAse digestion. The digest products are visualized by denaturing PAGE (119). This method has been used to detect K-*ras* mutations in colon cancer (120). A variation of this method employs DNA probes, and relies upon the formation of DNA:RNA hybrids prior to RNAse digestion (82). The location or type of mutation can not be identified without further testing.

Immunohistochemical Staining

This author found one report of immunohistochemical staining applied to the detection of mutations in the K-*ras* gene (121). The method used anti-ras (rap 5) antibodies to detect the presence of the K-ras protein on slides prepared from tissue. The staining was scored by visual analysis of the distribution of stained cells, as well as the intensity of staining. The authors assumed that activating point mutations resulted in overexpression of the ras protein, and did not verify the presence of mutations or determine their types or locations.

Detection Methods and the Reported Incidence of Mutations

The incidence, locations, and types of mutations reported depends upon the methods used for detection as well as the selection of the sample population. Table 5 is a summary of the results obtained using the above described methods.

Materials and Methods

Of the above methods, dot blot analysis and AS-PCR were used by this author to detect mutations in the K-*ras* gene because of their ability to detect and characterize mutations without additional testing, reportedly without the use of DNA enrichment methods. The adaptations employed will be described here.

Table 5

Incidence of K-*ras* Mutations in Colorectal Cancer by Type of Detection Method

Reference Number	Detection Method	Total Mutations Number	%	% in Codon 12 Asp	Val	Ala	Ser	Arg	Cys	% in Codon 13 Asp	Val	Ala	Ser	Arg	Cys	Multiple Mutations Or Comments
(62)	Sequencing	42/119	35	21	21	14	3	5	10	26	0	0	0	0	0	0
(71)	Sequencing	21/68	31	24	19	19	0	0	0	28	5	0	0	0	0	5% Asp^{12}-Asp^{13}
(63)	Sequencing	25/70	36													
(73)	Sequencing	19/37	51	48	26	0	5	0	0	21	0	0	0	0	0	0
(84)	Sequencing	90/247	36	31	20	13	6	1	7	22	0	0	0	0	0	0
(122)	PCR-SSCP	39/43	42													
(36)	PCR-SSCP	4/13	31													
(72)	PCR-SSCP & Sequencing	38/73	52	21	26	3	11	0	5	0	0	0	0	3	0	18% Val^{12}+Ala^{12} 5% Val^{12}+Cys^{12} 8% Val^{12}+Ala^{12}+Cys^{12}
(83)	PCR-Slot Blot	48/129	37	25	27	8	13	8	17	2						
(38)	PCR-Dot Blot	9/26	35	67	11	0	0	0	11							5% Asp^{12}+Val^{12}

Table 5--Continued

Reference Number	Detection Method	Total Mutations Number	%	% in Codon 12 Asp	Val	Ala	Ser	Arg	Cys	% in Codon 13 Asp	Val	Ala	Ser	Arg	Cys	Multiple Mutations/ Comments
(123)	PCR-Dot Blot	9/24	38													
(35)	PCR-Dot Blot	17/65	26													79% in codon 12 21% in codon 61
(13)	PCR-Dot Blot	38/92	41	29	16	5	13			16	18					3% His^{61} (16% codon 12 + 13)#
(33)	PCR-Dot Blot	10/29	34	70	30											
(104)	ME-RFLP	25/65	38													Only tested codon 12
(77)	ME-RFLP	6/10	60													Only tested codon 12
(109)	ME-RFLP	98/244	40													Only tested codon 12
(108)	ME-RFLP & Sequencing	4/15	27	75	0	0	0	0	0	25						
(111)	ME-RFLP & Sequencing	75/175	43													Only tested codon 12
(51)	ME-RFLP	5/11	45													2-stage-- codon 12

Table 5--Continued

Reference Number	Detection Method	Total Mutations Number	%	% in Codon 12 Asp	Val	Ala	Ser	Arg	Cys	% in Codon 13 Asp	Val	Ala	Ser	Arg	Cys	Multiple Mutations Or Comments
(70)	ME-RFLP	26/60	43													2-stage-- codon 12
(43)	ME-RFLP	46/92	50													2 stage--codon 12
(42)	ME-RFLP	32/42	76													2-stage--codon 12
(67)	AS-PCR	25/35	71*	+	+	+	+	+	+	+	+	+				Multiplex screen
(113)	AS-PCR	17/43	40	41	6	0	6	0	24	24	0	0				Multiplex screen
(65)	AS-PCR	2/9	22	0	0	0	0	0	50	50	0	0				
(118)	Ligation Assay	48/110	44													
(116)	Ligation Assay	5/10	50													
(120)	RNAse Cleav.	26/66	39													31% in 12, position 1 69% in 12, position 2
(121)	Immunohisto.	20/55	36													

Legend: Blank = Not tested, + = Reported finding, did not report incidence, * = All recurrent, # = Specifics not reported

Dot Blot Analysis

Oligonucleotide probes labeled with biotin (for non-isotopic detection) were purchased from National Biosciences, Inc. (NBI). The probe sequences, which analyze all possible single point mutations in K-*ras* codons 12, 13, and 61, have been previously described (100).

DNA was extracted from archival tumor samples using the modified method 14 (Chapter II, pages 29-30). A PCR mastermix was prepared and distributed into thin-walled reaction tubes, 75 µl per tube. Twenty-five µl of DNA were added just prior to cycling, bringing the final reaction volume to 100 µl (final reaction conditions: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.8 µM each primer, 4.0 mM $MgCl_2$, 200 µM each dNTP, 2.5 Units *Taq*). The tubes were gently mixed by tapping and overlaid with mineral oil. Amplification was performed in a Perkin Elmer model 480 thermocycler. The amplification program consisted of an initial denaturing step for 5 minutes at 95°C, 30 cycles of PCR, and a final extension of 10 minutes at 72°C. Each PCR cycle consisted of 1 minute at 95°C, 2 minutes at 52°C, and 1 minute at 72°C.

Ten µl of each PCR product were electrophoresed in a 2.0% Seakem GTG agarose gel for 40 minutes at 80 volts, and visualized with ethidium bromide staining and UV epi-illumination to verify the presence of PCR product of the appropriate size. Seventy-five µl of each PCR product were purified using Qiagen's QiaQuik PCR Purification Kit, following packaged instructions.

The denatured, purified PCR product was vacuum blotted onto a nylon membrane using a BioRad dot blot apparatus. The blot was dried for 30 minutes at 80°C, and stored in a sealed container between two sheets of filter paper until use. Hybridization of biotin-labeled probes (50 μg/1 ml) was performed at 56°C using the Oncogene Sciences Hybridization System according to packaged instructions. Post-hybridization washes (low and high stringency) were also used as instructed.

A chemiluminescent detection method was used, which employs the substrate CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'chloro)tricyclo[3.3.1.$1^{3,7}$] decan}-4-yl)phenyl phosphate) (Boehringer Mannheim catalogue number 1655 884). Following the final post-hybridization wash, the membranes were washed in TBS-Tween 20 (100 mM Tris, 150 mM NaCl, 0.05% Tween 20 (v/v), pH 7.5) for 2-3 minutes at room temperature, followed by blocking with 3% bovine serum albumin fraction V (Gibco catalogue number 11018-017) in TBS-Tween 20 for one hour at 65°C.

After removal of the blocking agent by rinsing with TBS-Tween 20, the blot was covered with streptavidin-alkaline phosphatase conjugate (Gibco catalogue number 19542-018), 1 μg/ml in TBS-Tween 20, and incubated for 10 minutes at room temperature. The blot was washed three times with TBS-Tween 20 (5 minutes each wash), and twice with detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5). The blots were spread with CSPD substrate diluted in the same detection buffer, and heat sealed in a plastic sheet protector. After exposing the blot to Kodak X-ray film for 15-30 minutes, the film was processed using Kodak D19

developer and Kodafix fixative.

Reverse Dot Blot Analysis

With the reverse dot blot method, unlabeled oligonucleotide probes were blotted onto the membrane. PCR was performed using biotin-labeled primers, and the PCR products were hybridized to the probes on the membrane. The blotting, hybridization, and detection were performed as described above except that 50 ng of unlabeled probes were blotted onto the membrane in an array which allowed for separate sections of the blot to be used for hybridization with each sample. Also, 75 µl of biotin-labeled PCR product was used in 10 ml of hybridization buffer.

Hybridization in a 96-Well Format

The reverse dot blot method was adapted to a 96-well format, using Nunc CovaLink NH microwell plates (124). With these plates, secondary amino groups are positioned at the end of a linker, which is attached to the bottom of the wells. Single-stranded DNA can be covalently bound exclusively at the 5' end by a phosphoramidite bond (125). Denatured, unlabeled probes were applied to the plate as described by the authors, 500 ng/well.

Biotin-labeled PCR products were denatured and hybridized to the coated plates using the Roche Amplicor hybridization system. All components for denaturation, hybridization, and detection were supplied ready-to-use. Washing buffer was diluted as instructed. The product was used as described, except that the

plates were sealed to prevent evaporation during hybridization, which was performed overnight at 56°C.

Post-hybridization washing was performed using a BioTech microplate washer. A streptavidin-horseradish peroxidase conjugate (SA-HRP) was added, allowed to bind the biotin, and the excess washed away. The biotin-bound SA-HRP was detected using the substrate tetramethylbenzidine (TMB), which produces a blue color when oxidized by HRP. The development was halted with the addition of the stop agent containing dilute HCl, which changed the color of the solution to yellow. The plate was read in a BioTech model 340 microplate reader at the prescribed wavelength of 450 nm. Absorbance readings were compared to known normal and abnormal results.

Allele-Specific Polymerase Chain Reaction

For this study, a new approach for this method was developed, which made it possible to detect any combination of mutations in codons 12 and 13. With this approach, codon twelve was analyzed in one direction, while codon 13 was analyzed in the opposing direction. The outside positions (position 1 of codon 12 and position 2 of codon 13) were analyzed during the first round of amplification. Then, taking the results of the first round into account, the inside positions were analyzed (position 2 of codon 12 and position 1 of codon 13). Figure 8 demonstrates this approach.

The design requires a minimum of 16 amplification reactions to analyze all possible mutations in both codons. In order to accomplish this in an efficient manner,

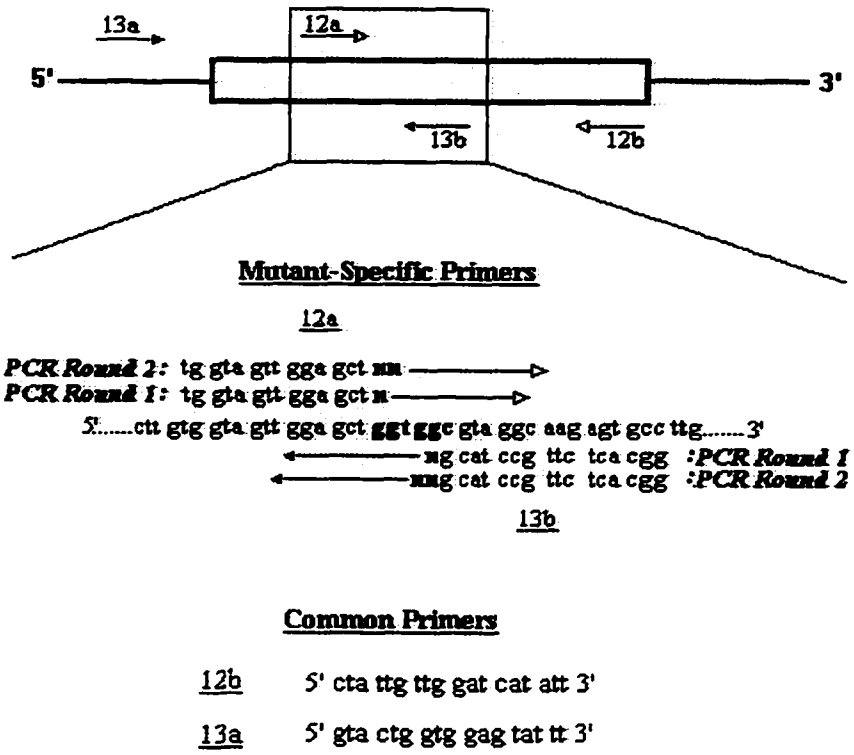


Figure 8. AS-PCR Opposing Primer Design for Codons 12 and 13.

the method was applied to a 96-well format. This allowed for the use of an 8-channel pipettor for the addition of PCR mastermix, and arranged reactions of a single sample alongside each other for further ease in pipetting. The design is shown in Figure 9.

The 16 mutant-specific and two common oligonucleotide primers were synthesized on a Perceptive Biosystems Expedite 8905, using standard phosphoramidite chemistry. The ammonium hydroxide solution, used to elute the primers from their columns was evaporated in a centrifugal vacuum concentrator. The primers were resuspended in sterile water, and their concentrations and purity were determined by measuring absorbances at 260 and 280 nm on a spectrophotometer.

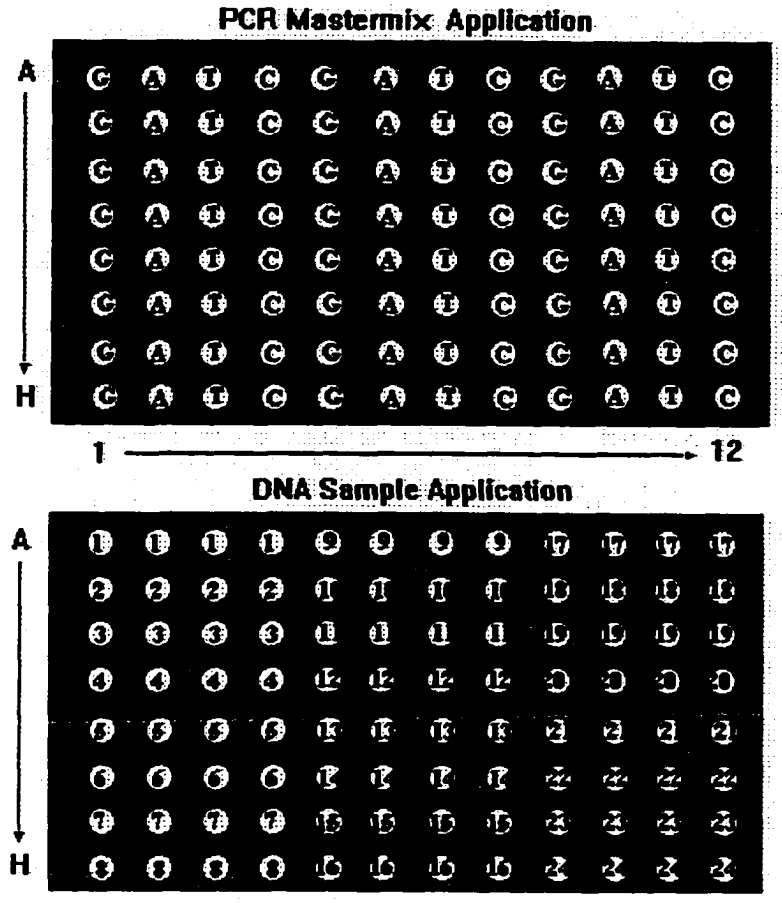


Figure 9. PCR Amplification in a 96-Well Format.

PCR optimization was performed as described previously, using a Taguchi array, except that 35 cycles of amplification were performed in 50 µl total volume (additional reaction conditions: 2.5 Units *Taq* DNA polymerase, 10 mM Tris-HCl pH 8.3, 50 mM KCl, and 0.5 µg of DNA extracted from fresh cells). No mineral oil overlay was used, and amplification was performed in a Perkin Elmer model 9600 thermocycler. DNA was denatured for 5 minutes at 95°C, followed by 35 cycles of PCR, and a final extension of 10 minutes at 72°C. Each cycle consisted of 30 seconds at 95°C, 60 seconds at 52°C, and 30 seconds at 72°C.

A commercially available PCR mastermix (Boehringer Mannheim catalogue number 1636 103) was found to have the same concentrations as were determined to be optimal, and was used in all subsequent experiments. A separate master mix was prepared for each set of primers to be used. Each master mix was then aliquoted into a 96-well tray, 40 µl per tube (final reaction conditions: 2.5 Units *Taq* DNA polymerase in Brij 35, 0.005% (v/v), 0.2 mM each dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 1 µM each primer). Ten microliters of DNA extract were added to each tube immediately before amplification, which was performed as described above.

PCR products were analyzed using microplate array diagonal gel electrophoresis (MADGE). The wells were formed in a diagonal 96-well array (using a 96-pin bacterial replicator tool) to facilitate gel loading, capacity and interpretation. Ten microliters of each PCR product were loaded onto the gel using a multichannel pipettor. Electrophoresis was performed in a 2.0% Seakem GTG agarose gel in Tris acetate EDTA (TAE) buffer containing ethidium bromide at 80 Volts for 40 minutes, allowing for maximum separation without loss of product into another well.

The gels were photographed using a Polaroid camera with UV epi-illumination. Presence of PCR product identified a perfect match at the 3' end of the mutation-specific primer, and thus identified the nucleotide at the corresponding position in the DNA. (With codon 13, the nucleotide identified was complementary to the 3' nucleotide of the primer.)

Results

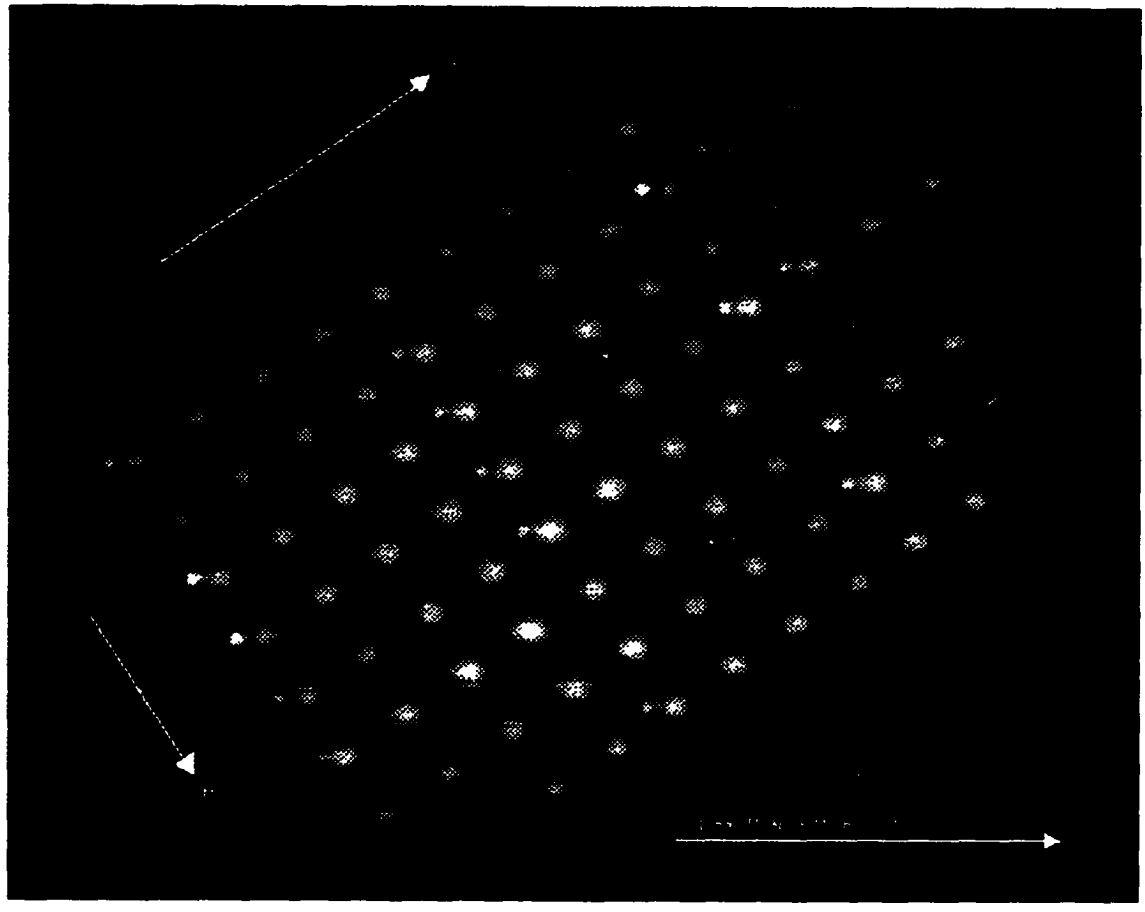
Hybridization Methods

All 14 patients who were analyzed with dot blot or reverse dot blot hybridization had essentially the same result. Although the background hybridization was greatly reduced with the reverse dot blot, single base discrimination was not accomplished. The adaptation to the 96-well format was promising, but only one tumor sample and two controls were processed, as the expense was found to be too prohibitive. Although the PCR product could be stripped, and the coated plate used again, the background increased with each trial.

Allele-Specific Polymerase Chain Reaction

The adaptation of AS-PCR used by this author proved to be highly successful, with all of the initial results being reproducible. A representative gel is shown in Figure 10, which shows 24 patients analyzed for position 2 of codon 13.

Note that there is the possibility of a mixed population of cells, or different mutations in each allele, resulting in multiple amplifications. The wild-type (guanine) reaction was used as an internal control, to assure that not only was there amplifiable DNA present, but that the gene in question was amplifiable. A negative result for the mutation specific reactions was only accepted if the wild-type reaction amplified for that position.



Legend: Mutant amplifications for adenine in samples 5, 11, 18 and 20.

Figure 10. Microwell Array Diagonal Gel Electrophoresis.

Discussion

It was this author's intent to utilize a method of mutation detection that would also serve to characterize the exact type and location of the mutation, eliminating the necessity for additional analysis. This eliminated several options, such as immunohistochemistry, RNAse mismatch cleavage, mutant-enriched restriction fragment length polymorphism, and single-stranded conformation polymorphism.

These methods are only able to detect possible mutations. Further testing, such as sequencing, would need to be employed to fully characterize the exact nature of the mutation, i.e., the location and type of the mutation.

The use of archival material embedded in paraffin made other considerations necessary. Only a minimal amount of each tissue sample could be used, in order to allow for future analysis of other genes from the same samples. Therefore, any method employed required the use of amplification. This eliminated the allele-specific ligation assay, which did not employ amplification as described (116-118).

Of the remaining methods, direct sequencing was the best characterized in the literature. However, this method was reportedly not able to detect mutations in tumor cells that make up less than 25% of the total cells (96). Since microdissection would not be employed to enrich for tumor cells, this level of sensitivity was not acceptable.

Hybridization Methods

At the time that this project was undertaken, dot blot analysis was the best characterized in the literature of the remaining methods. Although none of the twelve reports sited here discussed the sensitivity limit of this method, reports that single-base substitutions could be discriminated made the method attractive (100). However, this author found the method unable to detect mutations in a background of normal cells, as all samples analyzed yielded the same hybridization pattern.

Since high background hybridization in non-isotopic dot blot analysis made interpretation highly subjective, the author sought to use a less subjective method for

interpretation. The reverse dot blot method was adapted to a 96-well format, which allowed final results to be analyzed in a spectrophotometric microplate reader. Positive and negative results could then be assigned via numeric absorbance values.

In order to accomplish this, microplates needed to be coated with oligonucleotide probes. This presented a dilemma, since high stringency washes were required for single-base discrimination. However, if the probes were not firmly linked to the plate, they would wash away during this process. On the other hand, if stringency were reduced to avoid this, background would increase both due to PCR product adhering to the plate, and due to single base mismatches.

To solve this dilemma, a new product, the Nunc CovaLink NH plate was used. This plate had a linker arm with a secondary amino group bound to the surface. DNA probes were covalently linked to this amino group exclusively at the 5' end, using standard phosphoramidite chemistry as described by the manufacturer (125).

This method was satisfactory when used to evaluate the DNA sample and controls used to evaluate the protocol, however the cost of the plates was prohibitive. Also, this method was not able to detect multiple mutations in the same sample. Therefore, this approach was abandoned.

Allele-Specific PCR With MADGE Analysis

The mutant-specific amplification method described by Stork *et al.*(89), had potential for application to archived samples. Since amplification and enrichment occurred in a single-step, it was a very attractive option.

Nonetheless, the method as described had flaws. The opposing approach for codon 12 positions one and two seemed to solve the problem of a mutation in one position interfering with the detection of a mutation in the other position (Figure 6). However, there was no provision made for mutations in codon 13, and a mutation there would interfere with codon 12 position 2 detection. To resolve these issues, a new approach was developed, which made it possible to detect any combination of mutations in codons 12 and 13 (Figure 8).

A PCR optimization method employing a Taguchi's array was used for all amplifications during this project. Optimization experiments were performed using DNA from fresh cells, to assure adequate specificity. Some authors have reported decreasing the annealing temperature, or increasing the magnesium concentration in amplification protocols with archival samples in order to increase the sensitivity. However, either of these alterations decreases specificity, resulting in non-specific annealing and non-specific PCR product. This author did not find this appropriate (or necessary).

Conclusions

The adaptation of AS-PCR described by this author was determined to be the best method for the detection and characterization of mutations in the K-*ras* gene. Since enrichment for mutant sequences occurs during amplification, prior enrichment for tumor cells by microdissection is not required. The method described is not able to distinguish whether or not mutations detected concurrently in both codons 12 *and*

13 occurred together on the same strand of DNA. However, the method is able to distinguish whether or not two point mutations in the same codon (12 *or* 13) occurred together on the same strand of DNA, making the correct assignment of amino acids possible.

CHAPTER VI

ANALYSIS OF STUDY GROUP

Patient Population and Sampling Criteria

The study group consisted of patients who underwent surgery at Ferguson Hospital between 1970 and 1983, and were subsequently diagnosed with Dukes' Class B_2 or C_2 colorectal carcinoma according to the modified Dukes' classification (3). According to this staging system, patients staged as Dukes' B_2 and C_2 have tumors that extend through the bowel wall. Dukes' B_2 patients have no identified lymph node metastasis, while Dukes' C_2 patients exhibit metastasis in their regional lymph nodes. Complete follow-up with accurate survival data was available for all 97 patients from the Ferguson-Blodgett Digestive Disease Institute's colorectal database. (A detailed data entry sheet can be found in Appendix B.)

Patients were chosen who had short-term survival (less than 5 years, with death due to colon cancer), and who had long-term survival (greater than 10 years, disease free). Any patients who died of causes other than colorectal carcinoma, or who had other clinicopathological factors that may affect the data were excluded. The study group of 97 was divided into four cohorts: (1) 24 Dukes' Class B_2 patients with short-term survival (less than 5 years), (2) 24 Dukes' Class B_2 patients with long-term survival (greater than 10 years), (3) 24 Dukes' Class C_2 patients with short-

term survival and (4) 25 Dukes' Class C_2 with long-term survival.

All paraffin blocks and corresponding hematoxylin-eosin stained slides were obtained from the pathology archives of Ferguson Hospital. Hematoxylin-eosin stained slides were reviewed, and one paraffin block for each patient was chosen that exhibited morphology consistent with the diagnosis. All paraffin blocks containing lymph nodes were evaluated for each Dukes' B_2 patient who was found to have a K-*ras* mutation in the tumor. During the test period (1970-1983) mesenteric clearing of lymph nodes was practiced, assuring the examination of all regional lymph nodes in this study.

Sample Preparation

A single 10 micron-thick section was removed from each paraffin block using a disposable microtome blade, and placed into a sterile microcentrifuge tube. All instruments were thoroughly cleaned with chlorine bleach followed by ethanol, and gloves were changed between each sample to prevent cross-contamination. Sterile, ultrafiltered, deionized water was added to each tube in the volume of 100 µl. The tubes were sealed and heated at 95°C for 15 minutes, and briefly centrifuged to collect the sample into the bottom of the tubes, followed by cooling for 10 minutes at 4°C. The supernatant was transferred to a second sterile tube, and stored at -70°C until use.

K-*ras* Mutational Analysis

The tumor DNA extracts were analyzed using allele-specific polymerase chain reaction (AS-PCR) and microplate array diagonal gel electrophoresis (MADGE) as described beginning on page 48.

All Dukes' B_2 patients who were found to have mutations in their tumors were analyzed further. DNA was extracted from each block of lymph nodes and analyzed individually to determine if the same mutation could be identified.

Statistical Analysis

Statistical analysis was performed using SPSS software. Chi-squared analysis was used to determine whether or not differences exist in age, sex, surgeon, site of primary tumor, histopathological grade, Dukes' classification, and K-*ras* mutation incidence, location, or type between the two survival groups ($p < 0.05$).

CHAPTER VII

RESULTS

Characteristics of Sample

Complete data at all four nucleotides were obtained for 89 of the 97 patients tested (92%). The group of 89 patients consisted of 44 males and 45 females, with ages ranging from 34 to 88 years, and a mean age of 64 years. The tumors were located at the rectosigmoid junction in 68 patients and in the rectum in 22 patients. (One patient had synchronous lesions in both locations.) There were 45 Dukes' B_2 patients and 44 patients classified as Dukes' C_2 colorectal adenocarcinoma. The short-term survival group consisted of 42 patients, while the long-term survival group consisted of 47 patients. Using Chi-squared analysis, there were no significant differences found in age, sex, surgeon, site of tumor, histological grade, or Dukes' classification between the survival groups ($p < 0.05$).

The subgroup of Dukes B_2 patients consisted of 21 males and 24 females, with ages ranging from 34 to 88 years, and a mean age of 67 years. There were 20 patients with survival less than five years, and 25 patients with survival greater than 10 years. The tumors were located at the rectosigmoid junction in 36 patients and in the rectum in 10 patients. (One patient had synchronous lesions in both sites.) Chi-squared analysis revealed no significant differences found in age, sex, surgeon, site of tumor,

histological grade, or Dukes' classification between survival groups ($p < 0.05$).

Analysis of Mutations in K-*ras* Exon 1

Analysis of DNA From Tumors

AS-PCR was highly successful when applied to archival material, with 92% (89/97) of samples being amplified at all 4 nucleotide positions analyzed. In codon 12, there were 9 glycine→aspartic acid mutations, 6 glycine→valine mutations, 1 glycine→arginine mutation, and 1 glycine→cysteine mutation. In codon 13, there were 37 glycine→aspartic acid mutations, 4 glycine→serine mutations, 1 glycine→ cysteine mutation, and 1 glycine→alanine mutation.

Of these mutations, 4 patients had multiple mutations in the same codon (1 patient had valine and cysteine at codon 12; 1 patient had aspartic acid, serine, and cysteine at codon 13; and 2 patients had aspartic acid and serine at codon 13). Eleven patients had mutations in both codons (8 patients with aspartic acid at both codons, and 3 patients with valine at codon 12 and aspartic acid at codon 13).

There were no significant differences in mutational frequencies between tumor stages (Table 6). This table presents the total number of patients by mutation location, not the total number of mutations by location. (Note that this differs from the number of each type of mutation shown above. In cases with multiple mutations, the patient was counted only once for this table.) No significant relationship was found between survival and the presence or absence of a single mutation in either codon. However, colorectal cancers with concurrent mutations in codon 12 and 13

occurred significantly more frequently in long-term compared to short-term survivors (21.3% versus 2.4%).

Table 6

Patients With Mutations by Location

Group	None	Codon 12	Codon 13	Codons 12 & 13
B_2	51.1% (23)	2.2% (1)	35.6% (16)	11.1% (5)
C_2	50.0% (22)	9.1% (4)	27.3% (12)	13.6% (6)
< 5 Years	57.1% (24)	11.9% (5)	28.6% (12)	2.4% (1)*
> 10 Years	44.7% (21)	0% (0)	34.0% (16)	21.3% (10)*

* $p < 0.05$ Chi Square 5 year versus 10 year

Analysis of the types of mutations showed that glycine→aspartic acid mutations in either codon were related to long-term survival (Tables 7 and 8). (Totals for these tables were tallied as described for Table 6, above. If there were multiple mutations, and one was aspartic acid, the patient was counted as 'aspartic acid' and not as 'other mutation'.) Further, 8 of the 10 long-term survivors with mutations in both codons had aspartic acid substitutions in both codons.

Analysis of Dukes' Class B_2 Patients for Micrometastasis

Only Dukes' B_2 patients were analyzed further to detect micrometastasis. It was not necessary to analyze Dukes' C_2 patients, since metastasis was already

Table 7

Patients With Codon 12 Mutations by Type

Group	Aspartic Acid	Other Mutation	No Mutation
B_2	11.1% (5)	2.2% (1)	86.7% (39)
C_2	9.1% (4)	17.6% (6)	77.3% (34)
< 5 Years	2.4% (1) *	11.9% (5)	85.7% (36)
> 10 Years	17.0% (8) *	4.3% (2)	78.7% (37)

* $p < 0.05$ Chi Square 5 year versus 10 year

Table 8

Patients With Codon 13 Mutations by Type

Group	Aspartic Acid	Other Mutation	No Mutation
B_2	42.2% (19)	4.5% (2)	53.3% (24)
C_2	40.9% (18)	0% (0)	59.1% (26)
< 5 Years	31.0% (13)*	0% (0)	69.0% (29)
> 10 Years	51.1% (24)*	4.2% (2)	44.7% (21)

* $p < 0.05$ Chi Square 5 year versus 10 year

detected by histological examination of the lymph nodes.

DNA was successfully amplified at both codons in all 45 tumor specimens. Mutations were detected in 49% (22/45) of the specimens (Table 9). (Data are

presented as described for Table 6 above.) In codon 12, there were 5 glycine→ aspartic acid mutations, 1 one glycine→valine mutation. In codon 13, there were 19 glycine→aspartic acid mutations, 4 glycine→serine mutations, and 1 glycine→ alanine substitution. Three patients had multiple mutations in codon 13 (1 patient had aspartic acid, serine, and cysteine; and 2 patients had aspartic acid and serine), while 5 patients had aspartic acid mutations at both codons.

Table 9

Dukes' B_2 Patients With Mutations in Tumors by Location

Group	None	Codon 12	Codon 13	Codons 12 & 13
< 5 Years	65.0% (13)	5.0% (1)	30.0% (6)	0% (0)
> 10 Years	40.0% (10)	0% (0)	40.0% (10)	20.0% (5)

Of the 22 patients identified with mutation-positive tumors, mutational status of lymph nodes was evaluated in 20. (One patient was eliminated due to lack of archived lymph nodes, and amplifiable DNA was not obtained from a second patient's lymph nodes.) This group of 20 patients consisted of 11 males and 9 females, with ages ranging from 52 to 83 years, and a mean age of 65. Tumors were located at the rectosigmoid junction in 16 patients, and in the rectum in 4 patients. There were 6 patients in the short-term survival group, and 14 patients in the long-term survival group.

The incidence of mutations in the lymph nodes in this group was 80% (16/20)

(Table 10). (Data for Table 10 are presented as described for Table 6 above.) In codon 12, there was 1 glycine→aspartic acid and 1 glycine→valine mutation. In codon 13, there were 15 glycine→aspartic acid and 2 glycine→serine mutations. Two patients had multiple mutations in codon 13 (aspartic acid and serine), and 1 patient had aspartic acid mutations at both codons.

Table 10

Dukes' B_2 Patients With Mutations in Lymph Nodes by Location

Group	None	Codon 12	Codon 13	Codons 12 & 13
< 5 Years``	0% (0)	10.0% (1)	90.0% (5)	0% (0)
> 10 Years	28.6% (4)	0% (0)	64.3% (9)	7.1% (1)

All 4 patients with mutation-free nodes were disease-free after 10 years (mean survival at last follow-up = 19 years), while 37.5% (6/16) of the patients with mutation-positive nodes died as a result of their disease within five years. (Statistical analysis was not performed, due to the small sample size.)

CHAPTER VIII

DISCUSSION

K-*ras* Tumor Status as a Prognostic Marker

The results of this study are consistent with the reported incidence of K-*ras* mutations in colorectal cancers of 40-60%. The most frequent mutations appear to be those resulting in the substitution of aspartic acid for glycine at either codon 12 or 13, as others have also reported (62,71,84,113).

Benhattar *et al.* (78) reported a significantly higher incidence of mutations in recurrent (71%) versus disease-free patients (25%), and mutations other than glycine→aspartic acid were correlated with very high risk for recurrence, indicated that replacement by aspartic acid was related to longer survival than replacement by other amino acids. Although the findings in the current study did not correlate amino acid substitution other than aspartic acid with poor survival, the data did show survival advantages with aspartic acid rather than other amino acids (Tables 7 and 8).

The differences between the current study results and those of Benhattar *et al.*(78) may be attributed to differences in the selection of sub-groups, i.e., the selection of patients who were obviously cured of the disease (greater than 10 year survivors) and those who lived for less than five years may have exaggerated differences in biological tumor aggressivity. Like the current study, Benhattar *et al.*

employed AS-PCR for mutation analysis. However, they used a unidirectional approach that would not have allowed the detection of a double mutation.

Finkelstein *et al.* (84) reported that tumor recurrence patterns seem to correlate with K-*ras* mutation patterns—that is, local recurrence was associated with lower incidences of mutation compared to tumors complicated by hematogenous spread. They also identified aspartic acid substitutions at codon 13 as prognostically favorable (62,84). However, a codon 12 mutation resulting in aspartic acid or any other amino acid correlated with a very poor prognosis (62,84).

The data from the present study showed similar results to the genotyping of tumors performed by Finkelstein (84), with aspartic acid substitution being the most frequent alteration at either codon. The results in Table 8 agree with Finkelstein's finding that mutations resulting in aspartic acid substitutions at codon 13 were prognostically favorable. However, in the current study no significant differences were identified in the incidence of K-*ras* mutations with respect to survival status (less than five year--43%; greater than ten year--55%), or between Dukes stages (B_2--49%; C_2--50%).

Unlike the Finkelstein (83) report, with the use of more locally advanced tumors that differed only by the presence or absence of lymph node metastases (Dukes B_2 and C_2), the effect of isolated hematogenous spread of the disease was not evaluated here. Also, the current study identified a significantly higher incidence of aspartic acid substitutions at codon 12 in the greater than 10-year survivors compared to those with early recurrence (Table 7). However, this finding has been reported

before by Moerkerk *et al.*(72). They reported that, in a group of Dukes' class B and C colorectal cancers, aspartic acid at either codon was not associated with metastasis, whereas alanine or cysteine at codon 12 or arginine at codon 13 was associated with metastasis.

The current project identified an additional subset of patients with concurrent mutations at codon 12 and 13 in the long-term survivors group, with the most frequent pattern being replacement with aspartic acid at both locations (80%). Thus it appears that aspartic acid at codon 13 confers significant survival advantage to patients with colorectal cancer, which is enhanced by concurrent substitution of aspartic acid at codon 12.

Although the specific role that mutations in p21ras play in colorectal cancer development and subsequent tumor aggressivity remains unclear, there are several potential mechanisms of action. The net result of mutations at codon 12 or 13 is decreased intrinsic hydrolysis rate of GTP by 10-fold due to alterations in the spatial interaction with the phosphate groups (21), resulting in significant prolongation of protein activity. Alternatively, p21ras proteins that contain aspartic acid substitutions at codon 13 are capable of inducing T-lymphocyte response (126). Activation of these T-lymphocytes has been associated with a predominance of early stage carcinomas (127), which may account for the improved survival and lower recurrence rates in tumors with aspartic acid mutations at codons 12 and 13 identified both in the present study and by others (119).

The results of this study confirm that K-*ras* mutations occur in approximately

50% of sporadic colorectal carcinoma. The frequency of K-*ras* mutations did not differ between tumor stages (B_2-49%; C_2-50%), nor does the presence or absence of a mutation distinguish between patients with short term (43%) versus long-term (55%) survival. However, the presence of specific mutational patterns, particularly concurrent mutations leading to aspartic acid substitutions at codon 12 and 13, conferred a significantly higher likelihood of surviving longer than 10 years following resection of colorectal cancer.

K-*ras* Lymph Node Status as a Marker for Micrometastasis

There have been other methods used to identify micrometastases in lymph nodes of patients with colorectal cancer. Greenson *et al.* (128) and Cutait *et al.* (129) independently reported the use of immunohistochemical staining for cytokeratin as a means of identifying micrometastases in hematoxylin-eosin negative lymph nodes. Using this method, Greenson *et al.* (128) upstaged 28% of Dukes B colorectal cancers. However, their findings that micrometastases correlated to survival contradicted those of Cutait *et al.* (129), who found micrometastases in 26% of Dukes B patients, but found no correlation to survival.

Unlike cytokeratin staining, which is only able to distinguish that cells are of epithelial origin, the method reported here uses a specific pattern of mutations found in the tumor cells as a marker to identify micrometastasis. For this to be a valid indicator, histologically positive metastasis should have mutational patterns that match that of the associated tumor.

Losi, Benhattar, and Costa (67) reported that point mutations found in metastatic lesions, as well as those found in local recurrence, were identical to those found in the primary tumor. This was the case even when the recurrence was found up to five years after treatment for the primary cancer. Dix *et al.* (36) reported a similar finding in cases with lymph node and liver metastasis.

These findings, as well as evaluations of clonal progression within the primary lesions, support the use of oncogenic markers of disease progression (57). When heterogeneity was identified, it was in the form of an addition mutation at the point of deepest invasion, interpreted as a subclone of the original (38). However, even in these cases, the original mutation was also identified.

Together, these findings suggest that, even if further mutations occurred during progression of the disease, the mutation found within the tumor would be found in the metastatic lesions. It would, therefore, make sense to analyze the most common site of metastasis (the lymph nodes) for evidence of disease spread.

CHAPTER IX

CONCLUSIONS

This study confirmed that the presence of aspartic acid substitution at either codon 12 or 13 is a positive prognostic marker in colorectal cancer. Additionally, a new group was identified with concurrent mutations in both codons 12 and 13, which was significantly associated with long term survival.

Using K-*ras* mutation as an indicator of micrometastases, 35% (16/45) of Dukes' B_2 colorectal cancers were upstaged in the current study. Further, the absence of identified micrometastases by this method was a very favorable indicator of prognosis, with no recurrence after an average of 19 years. Since mutations occurred in 49% (22/45) of the Dukes' B_2 tumors, the method described here provides a tool to further evaluate approximately half of all Dukes' B colorectal cancers.

The evaluation of mutational status of other genes along with K-*ras* would provide a molecular 'fingerprint' of the tumor, which could be used to more clearly identify micrometastases in all Dukes' B colorectal cancers. Since K-*ras* and/or *p53* mutations have been found to occur in the vast majority of colorectal cancers (95,111), *p53* would be a good candidate for additional screening.

The author suggests expanding the study to a larger group for confirmation of the findings, followed by prospective studies to evaluate the benefit of additional treatment for Dukes' B patients identified as high risk by this method.

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Appendix A

Q-Basic Program for PCR Optimization

```
1   REM    ************PROGRAM FOR PCR OPTIMIZATION************
2   REM    CALCULATION OF SIGNAL TO NOISE RATIO (SNL) FOR PCR REACTION COMPONENTS
3   REM    THE OPTIMAL LEVEL FOR A COMPONENT IS THE LEVEL YIELDING THE HIGHEST SNL

11  PRINT  "************PCR OPTIMIZATION USING MODIFIED TAGUCHI METHOD************"
12  PRINT
13  PRINT  "THIS PROGRAM WILL COMPUTE THE SIGNAL TO NOISE RATIO FOR A SINGLE"
14  PRINT  "     COMPONENT AT THREE LEVELS AND CHOOSE THE OPTIMAL LEVEL."
15  PRINT  "IT ASSUMES THAT FOUR COMPONENTS WERE TESTED AT THREE LEVELS EACH."
16  PRINT

21  PRINT  "                YOU WILL BE ASKED TO INPUT THREE VALUES."
22  PRINT  "THESE MUST CORRESPOND TO THE DNA YIELDS OF THE PCR REACTIONS"
23  PRINT  "     IN WHICH THIS COMPONENT WAS TESTED AT THE LEVEL SHOWN."
24  PRINT

31  PRINT  "INPUT THE FIRST YIELD AT LEVEL 1"
32  INPUT  A1
33  PRINT  "INPUT THE SECOND YIELD AT LEVEL 1"
34  INPUT  B1
35  PRINT  "INPUT THE THIRD YIELD AT LEVEL 1"
36  INPUT  C1
37  PRINT

41  PRINT  "INPUT THE FIRST YIELD AT LEVEL 2"
42  INPUT  A2
43  PRINT  "INPUT THE SECOND YIELD AT LEVEL 2"
44  INPUT  B2
45  PRINT  "INPUT THE THIRD YIELD AT LEVEL 2"
46  INPUT  C2
47  PRINT

51  PRINT  "INPUT THE FIRST YIELD AT LEVEL 3"
52  INPUT  A3
53  PRINT  "INPUT THE SECOND YIELD AT LEVEL 3"
54  INPUT  B3
55  PRINT  "INPUT THE THIRD YIELD AT LEVEL 3"
56  INPUT  C3
57  PRINT

61  SNL1 = -10 * LOG (1 / 3 * (1 / (A1 * A1) + 1 / (B1 * B1) + 1 / (C1 * C1))) / 2.3
62  SNL2 = -10 * LOG (1 / 3 * (1 / (A2 * A2) + 1 / (B2 * B2) + 1 / (C2 * C2))) / 2.3
63  SNL3 = -10 * LOG (1 / 3 * (1 / (A3 * A3) + 1 / (B3 * B3) + 1 / (C3 * C3))) / 2.3

71  PRINT  "THE SIGNAL TO NOISE RATIO AT LEVEL 1 = ", SNL1
72  PRINT  "THE SIGNAL TO NOISE RATIO AT LEVEL 2 = ", SNL2
73  PRINT  "THE SIGNAL TO NOISE RATIO AT LEVEL 3 = ", SNL3
74  PRINT

81  IF SNL1 > SNL2 AND SNL1 > SBL 3 THEN
82  PRINT  "THE OPTIMAL LEVEL FOR THIS COMPONENT IS LEVEL 1"
83  ELSEIF SNL2 > SNL1 AND SNL2 > SNL3 THEN
84  PRINT  "THE OPTIMAL LEVEL FOR THIS COMPONENT IS LEVEL 2"
85  ELSE PRINT "THE OPTIMAL LEVEL FOR THIS COMPONENT IS LEVEL 3"
86  END IF

91  END
```

Appendix B

Ferguson Research Department
Data Collection Worksheet

COLORECTAL CANCER
Data Entry Form

Demographics

Last Name _____ First Name _____

Address _____

City, State, Zip _____

Area Code _____ Phone # _____

Birthdate _____ Hosp # _____

Sex M F

Race White/Caucasian Black/African American Hispanic/Latin Asian/Asian American
American Indian/Alaskan Native Other Not Stated Multiracial

Date Diagnosed with CRC _____

Pre-existing Medical Conditions

Thyroid	Hypertension	Cardiac Arrhythmia
CUC	MI	CVA/TIA/Stroke
Crohn's	CHF	COPD
Diabetes	Heart Disease	Prior Cancer
Cirrhosis	Bleed Disorder	Cancer after Primary

List Other Pre-existing _____

Follow Up

Last follow-up date _____ If dead, was autopsy done? Y N

Death Y N Date Last Letter Sent _____

Death due to CRC? Y N Unknown

Death Cause Hemorrhage Cardiac Sepsis Other

If other list cause _____

Visits

Date Reviewed _____ Date of Visit _____

Account # _____

Age _____

Classification of CRC Visit

Primary CRC
Metachronous CRC
Recurrent CRC
Appendiceal Primary
Metachronous non-CRC
Other, Please specify _____
Lap (see lap instructions)
GI Bleed (Embolization Study)
Fresh Tissue, pending
Primary non-CRC
Recurrent non-CRC

Type of Visit

SURGICAL admission. Hosp. chart available
SURGICAL admission. Hosp. chrt not available
MEDICAL admission. Hosp chrt available
MEDICAL admission. Hosp. chrt not available
OFFICE visit/Telephone f/u
DIAGNOSTIC testing independent of office visit

Hospital

Yes – Ferguson Clinic chrt
No – Ferguson Clinic chrt
BMMC
BWH
SMH
Metro
Other _____

Principal Surgery

Rt. Hemi
Lt. Hemi
Total Colectomy
Anterior/Sigmoid Resection
Low Anterior Resection
Low LAR
Mid LAR
High LAR
APR
None – Unresectable
TRE, Fulguration
Colotomy, Polypectomy
Bx. Only (refuse surg)
Small Bowel Resection
Hartmann
Ostomy Closure
Ostomy Formation
J Pouch
Proctocolectomy
Proctectomy
Subtotal Colectomy
S Pouch
Other Surgery_____(include appendix,gallbaldder,etc.)

Ferguson Clinic Surgeon

Mazier
MacKeigan
Talbott
Luchtefeld
Senagore
Surrell

General Surgeon

Post, Kenneth
Zadvinskis, Ivars
Wilcox, Richard
Johnson, Greg
DeVries, Daniel
Rodriguez, Carlos
Lovell, Raymer
Stawski, Willard
Sherman, Stanley
Gladding, Sandra
Borreson, Daniel
Passinault
Other surgeon_____

Surgery Variables

Colostomy Y N Blood Loss _____(ml or cc) *op report

Ileostomy Y N Brook Continent (Kock,Barnett)

Elective Emergent (circle)

Anastomic Technique

Stapled
Stapled EEA
Stapled GIA
HS 1 layer - interrupted
HS 1 layer - continuous
HS 2 layer - interrupted
HS 2 layer - continuous
HS 2 layer - inter & continuous
HS 1 layer - inter & continuous
No Anastomosis (colostomy,hartmann)
S/S Hand Sewn
E/E Hand Sewn
E/S Hand Sewn

Surgery, Other

Posterior Vaginectomy
Hysterectomy
Oophorectomy
Pelvic Exenteration

Tumor Extension at Surgery (op report)

Residual Tumor
Liver Metastasis
Peritoneal Spread

Time Study

Emergency Room Admit Y N Critical Path ☐

Date of Admit _____ Time of Admit _____

Date of surgery _____ Time Proc Start to Proc End _____(case log)

Date of Discharge _____ Time of Discharge _____

Hematology

CEA Preop _____ CEA Postop _____
(2 wks ≤ x ≤ 3 mo)

Transfusions

Did patient receive a blood transfusion? Y N If No, do not answer any of the questions below.

Preop Y N (2 days <x<1wk preop)

Periop Y N (± 2 days)

Postop Y N (>2 days postop)

(Total units) Platelets _____
PRBC's _____
Plasma _____

Postop Complications

UTI
Atelectasis
Pneumonia
Cardiac Arrythemia
MI
Sciatica
Pulmonary Emboli
Renal Failure
CHF/Cardiac Failure
Wound infection _____(site)
Dehiscence
Ileus
Bowel Obstruction
Inop Comp_____(site)
TIA/CVA/Stroke
Postop Bleed
Septicemia_____(site)
Respiratory Distress
Intrabd. Abscess
Stenosis
Anastomotic Leak
DVT
Other_____

Preop Diagnostics

Barium Enema
Flex Scope
Colonoscopy
Biopsy
Sigmoidoscopy
Retinoscopy
EGD
UGI
Abdomen
CT
IVP
Ultrasound
IRUS
Gastrograffin
Other _____

Anesthesia

ASA status (circle one) I II III IV V EI EII EIII EIV EV

Type of Analgesia General General/Epidural Spinal

Postop Analgesia (Circle all that apply for the FIRST THREE DAYS POSTOP) IM PCA EPI

Pathology I

Path Number _____
Any hx of metachronous lesions? Y N
# of Synchronous (more than one) colorectal CA's _____

Site One
Fresh tissue Y N
Distance from anal verge(cm)_____

Site Two
Distance from anal verge(cm) _____

*(see definitions below)

*Site 1 _____
List *other* site

*Site 2 _____
List *other* site

* CECUM	ASC, RT, HEPATIC FLEX	TRANSVERSE	DESC, SPLENIC FLEX	SIGM, RECTOSIGMOID
RECTUM	ILEUM	POUCH	STOMACH	DUODENUM	DUODENAL PAPILLA	*Other*	NONE

*Histology _____
List *other* Hist

*Histology_____
List *other* Hist

*ADENOCARCINOMA	ADENOMA - NOT SPECIFIED	TUBULAR ADENOMA	TUBULO VILLOUS ADENOMA
VILLOUS ADENOMA	LYMPHOID	HYPERPLASTIC/METAPLASTIC	FUNDIC GLAND/CYSTIC
HAMARTOMA	*Other*		

*Histologic Grade _____

*Histologic Grade_____

*Well Moderately Poorly Undifferentiated

(Site 1)

Total Nodes Found_____
Total Nodes Positive _____

Vessel Invasion
Nerve Invasion
Mucin Production
Lesion Size _____(cm)

(Site 2)

Total Nodes Found _____
Total Nodes Positive _____

Vessel Invasion
Nerve Invasion
Mucin Production
Lesion Size _____(cm)

PLEASE NOTE IN **COMMENTS** IF LYMPHANGITIC INVASION IS NOTED AND NODES POSITIVE = O

Site Three

Distance from anal verge(cm) _____

*(see definitions below)

*Site 3 _____
List *other* site

*CECUM ASC, RT, HEPATIC FLEX TRANSVERSE DESC, SPLENIC FLEX SIGM, RECTOSIGMOID
RECTUM ILEUM POUCH STOMACH DUODENUM DUODENAL PAPILLA *Other* NONE

*Histology _____
List *other* Hist

ADENOCARCINOMA ADENOMA - NOT SPECIFIED TUBULAR ADENOMA TUBULO VILLOUS ADENOMA
VILLOUS ADENOMA LYMPHOID HYPERPLASTIC/METAPLASTIC FUNDIC GLAND/CYSTIC
HAMARTOMA *Other*

*Histologic Grade _____

*Well Moderately Poorly Undifferentiated

Total Nodes Found _____
Total Nodes Positive _____

Vessel Invasion
Nerve Invasion
Mucin Production
Lesion Size _____(cm)

Site Four

Distance from anal verge(cm) _____

*Site 4 _____
List *other* site

*Histology _____
List *other* Hist

*Histologic Grade _____

Total Nodes Found _____
Total Nodes Positive _____

Vessel Invasion
Nerve Invasion
Mucin Production
Lesion Size _____(cm)

Are there any additional sites not entered? **Y N**

TNM Classification

TX: Primary tumor cannot be assessed, ie. no specimen
T0: No evidence of primary tumor, ie. no cancer found in specimen
Tis: Carcinoma in situ , ie. cancer in polyp, no invasion into wall
T1: Invades mucosa, submucosa, ie. Duke's A
T2: Invades into, but not through muscularis propria, ie. Duke's B1,C1
T3/T4: See below

***Serosa* (small bowel, colon, proximal (upper) one-third of rectum)**

T3: invades through muscularis propria (B2,C2)
- -into subserosa
- -into, but not through serosa
- -into pericolic fat, perirectal fat, soft tissue

T4: invades through serosa
- -into free peritoneal cavity
- -into contiguous\adjacent organ (vagina,prostate,ureter,kidney)

***No Serosa* (distal (lower) two-thirds of rectum)**

T3: invades through muscularis propria (B2,C2)
- -into pericolic fat, perirectal fat, soft tissue

T4: invades into contiguous/adjacent organ (vagina,prostate,ureter,kidney)

NX: Regional lymph nodes cannot be assessed, ie. no specimen
N0: No regional lymph node metastasis, ie. no positive nodes, Duke's A or B
N1: 1 to 3 positive pericolic or perirectal lymph nodes, ie. Duke's C
N2: 4 or more positive pericolic or perirectal lymph nodes, ie. Duke's C
N3: Positive/"enlarged" lymph nodes along the course of a named vascular trunk, ie. along the aorta (periaortic), celiac axis. Positive apical/apex nodes should be referred to AJS for staging

---

MX: Distant metastasis cannot be assessed. Pathologist usually stages as MX because there is no biopsy or tissue to stage. Distant metastasis should be assessed based on intraoperative findings, CT reports, x-rays, etc.
M0: No distant metastasis
M1: Distant metastasis, ie. liver, lung, INCOMPLETE excision/resection of peritoneal spread, small bowel mesentery, etc. Duke's D. Any spread in question should be referred to AJS.

Duke's A ($T_1N_0M_0$)
Duke's B_1 ($T_2N_0M_0$)
Duke's B_2 ($T_3N_0M_0$)
Duke's B_3 ($T_4N_0M_0$)

Duke's C_1 ($T_2N_1M_0$)
Duke's C_1 ($T_2N_2M_0$)
Duke's C_2 ($T_3N_1M_0$)
Duke's C_2 ($T_3N_2M_0$)

Duke's C_3 ($T_4N_1M_0$)
Duke's C_3 ($T_4N_2M_0$)
Duke's D ($T_?N_?M_1$)

(site 1)
Flow Cytometry Y N

(site 2)
Flow Cytometry Y N

NG/Diet/Bowel Functions

NG

NG inop and stays? Y N Date removed _____

NG inserted postop? Y N Date inserted _____ Date removed _____

Diet

Bowel Functions

Date Clear liquids _____

Date Flatus _____

Date Full liquids_____

Date BM _____

Date General/Post-Surgical Soft _____

If not stated, please make note in comments.

Temperature

MIN POD #1
MAX POD #1

MIN POD #2
MAX POD #2

MIN POD #3
MAX POD #3

MIN POD #4
MAX POD #4

MIN POD #5
MAX POD #5

Treatment for CRC *hx, phy exam

XRT for primary disease? Preop or Postop None Preop Postop Preop & Postop

XRT Dosage (cGY)_____

XRT after recurrence? Y N

Chemotherapy for primary disease? Preop or Postop None Preop Postop Preop & Postop

Chemo after recurrence? Y N

Treatment for Non-CRC

XRT for Non-CRC Y N Chemo for Non-CRC Y N

List Site _____

Additional Data Collection for Laparoscopic Patients!

Technique: Lap Converted Lap

Indication:
Adenocarcinoma
Diverticular Disease
Appendicitis
GI Bleed
Adenoma
Volvulus
IBD (Crohn's, CUC)
Rectal Prolapse
Other_____

Anastomosis:
Intracorporeal
Combined Intra/Extracorporeal

Assistant: **(please list)**

Intraoperative Comps:
Hemorrhage
Hypotension
Hypoventilation (? EtOH<=40)
Acidosis(1=metab 2=resp 3=mixed)
Enterotomy

Reasons for Conversion:
Adhesions
Bleeding
Perforation
Unfavorable Conditions

Pathology:
Lesion Length_____
Bowel Length_____

Crohn's
CUC
Appendicitis
Div-itis
Div-osis
Other Path_____

Other Postop Comps:
Laparotomy for Bow Obst

Other Surgical:
Ureteral Catheter

PHYSIOLOGICAL SCORE (preoperative evaluation)

(please write in actual values)

	1	2	4	8
Age (years)	≤60	61-70	≥71	
Cardiac signs	no failure	diuretic, digoxin, antianginal or hypertensive therapy	peripheral edema; warfarin therapy	aised jugular venous pressure
Chest X-ray			borderline cardiomegaly	cardiomegaly
Respiratory history	no SOB/dyspnea	SOB/dyspnea on exertion	SOB/dyspnea (one flight stairs)	SOB/dyspnea at rest (rate ≥30/min)
Chest X-ray		mild COAD (COPD) (chronic obstructive airway disease = chronic obstructive pulmonary disease)	moderate COAD	fibrosis or consolidation
Blood pressure (systolic-top) (mmHg)	110-130	131-170 100-109	≥171 90-99	- ≤89
Pulse (beats/min)	50-80	81-100 40-49	101-120	≥121 ≤39
Glasgow coma score	15	12-14	9-11	≤8
Hemoglobin (g/100ml) (Hgb)	13-16	11.5-12.9 16.1-17.0	10.0-11.4 17.1-18.0	≤9.9 ≥18.1
White cell count ($x10^{12}/l$) (WBC)	4-10	10.1-20.0 3.1-4.0	≥20.1 ≤3.0	
BUN (mg/dl) (mEq/L)	≤45 ≤7.5	46-60 7.6-10.0	61-90 10.1-15.0	≥91 ≥15.1
Sodium (mmol/l) (Na)	≥136	131-135	126-130	≤125

OPERATIVE SCORE (postoperative evaluation)

	1	2	4	8
Potassium (mmol/l) (K)	3.5-5.0	3.2-3.4 5.1-5.3	2.9-3.1 5.4-5.9	≤2.8 ≥6.0
Electrocardiogram(EKG) (EKG=ECG) (please write out results if unclear)	normal/ not done		atrial fibrillation (rate 60-90)	any other abnormal rhythm or ≥5 ectopics/min Q waves or ST/T wave changes
Operative severity	minor	moderate	major	major +
Multiple procedures	1		2	>2
Total blood loss (ml) (anesthesia)	≤100	101-500	501-999	≥1000
Peritoneal soiling (op report)	none	minor (serous fluid)	local pus or blood	free bowel content, pus
Presence of malignancy (path report)	none	primary only (no positive nodes)	nodal metastases (any positive nodes)	distant metastases (ie. liver mets)
Mode of surgery	elective		emergency resuscitation of >2h possible Operation <24h after admission	emergency (immediate <2h needed)

Appendix C

Molecular Data Collection Worksheet

Molecular Biology Laboratory
Ferguson-Blodgett Digestive Disease Institute
Research Department

K-ras mutation specific PCR results

		Codon 12 base		Codon 12 AA	Codon 13 base		Codon 13 AA	Comment
	Result	Position 1	Position 2		Position 1	Position 2		
Wild Type		G	G	Gly	G	G	Gly	
Sample #	first							
	second							
	third							
Sample #	first							
	second							
	third							
Sample #	first							
	second							
	third							
Sample #	first							
	second							
	third							
Sample #	first							
	second							
	third							
Sample #	first							
	second							
	third							
Sample #	first							
	second							
	third							
Sample #	first							
	second							
	third							
Sample #	first							
	second							
	third							
Sample #	first							
	second							
	third							
Sample #	first							
	second							
	third							

Appendix D

Ferguson Hospital Surgical Consent

FERGUSON
HOSPITAL
72 SHELDON BLVD. SE – GRAND RAPIDS, MICHIGAN 49503

PROCEDURE CONSENT FORM

1. I hereby authorize _____ M.D. and whomever he may designate as his assistant to perform the following contemplated procedure and/or procedures: _____ at Ferguson Hospital.

2. If any unforeseen condition(s) arises in the course of the procedure, I further request and authorize the physician to do procedures in addition to or different from those now contemplated, if in the physician's judgement he deems it advisable.

3. I acknowledge that the contemplated procedure, possible procedures in addition to the contemplated procedure, possible complications, and risks involved, have been explained to me by the physician to my satisfaction.

4. I acknowledge that alternative methods of treatment have been discussed with me by the physician to my satisfaction.

5. I acknowledge that no guarantee or assurance has been made as to the results that may be obtained. I understand that I am to have the usual and ordinary care practiced by physicians and surgeons and furnished by hospitals in this community and that no other promise or representation, written or implied, is made, nor is any employee or representative of the hospital authorized to make any other promise or representation.

6. I consent to the administration of sedation and/or anesthesia prescribed or chosen by the attending physician and/or surgeon and/or anesthesiologist.

7. I consent to photographs for educational purposes, provided my identity is not revealed, as my physician desires. I also consent to medical trainees being present in the room to observe for educational purposes.

8. I hereby authorize Ferguson Hospital to retain, preserve, and use for scientific or teaching purposes any specimen or tissue taken from my body.

9. I consent to the disposal by authorities of the Ferguson Hospital of any tissue or part which may be removed.

10. I ACKNOWLEDGE THAT I HAVE READ AND FULLY UNDERSTAND THE ABOVE CONSENT.

Signed _____
(patient)

Witness _____ or _____
(parent, guardian, other)

Date signed _____ Time _____ AM PM

_____
Relationship to Patient

0-18-601 (3/84)

Appendix E

Institutional Review Board Exclusion

Blodgett
MEMORIAL MEDICAL CENTER

MEMORANDUM

TO: Jennifer Thebo

FROM: Mary Ursul, General Counsel/Director of Administrative Services

DATE: March 18, 1998

RE: IRB EXCLUSION STATEMENT

I am in receipt of your memo dated March 17, 1998 regarding your research study entitled. "Molecular Staging of Colorectal Cancer: K-ras Mutation Analysis of Lymph Nodes Upstages Dukes' B_2 Patients." Because of the fact that this study utilizes archived material and previously collected patient data, it is not necessary for you to obtain IRB approval.

If I can be of any further assistance to you, please do not hesitate to contact me at 391-2729.

MLU/csd

150+ YEARS BLODGETT Caring For The Community 1840 Wealthy SE Grand Rapids MI 49506-2968 616-774-7444

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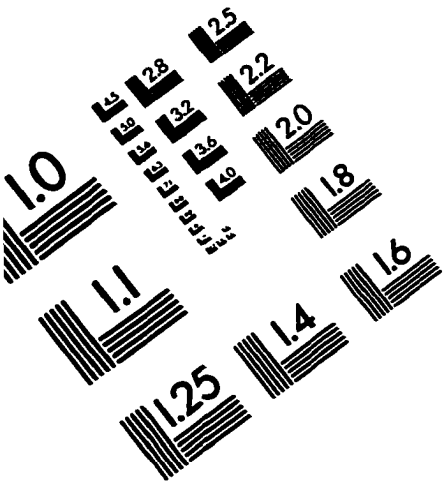
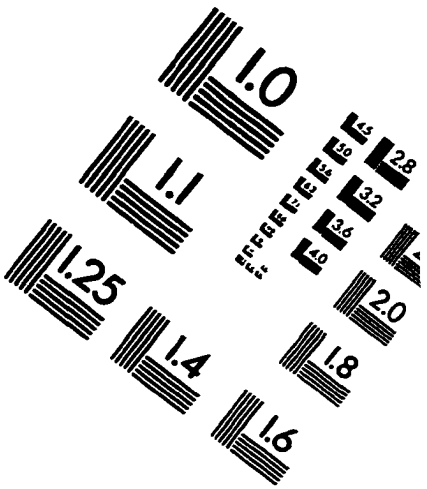
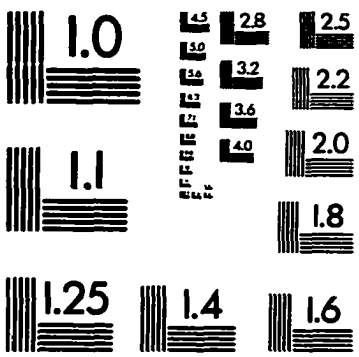
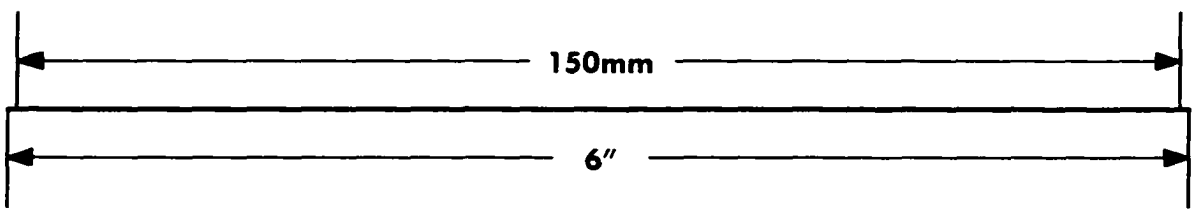
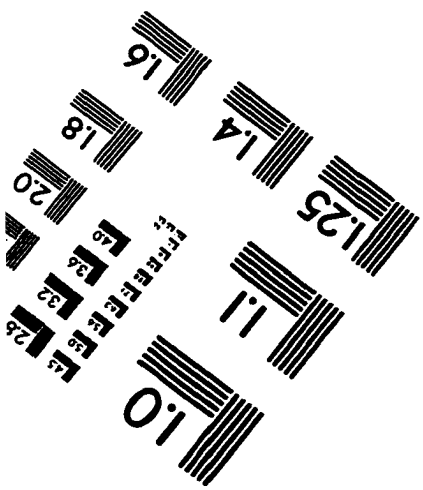
IMAGE EVALUATION TEST TARGET (QA-3)

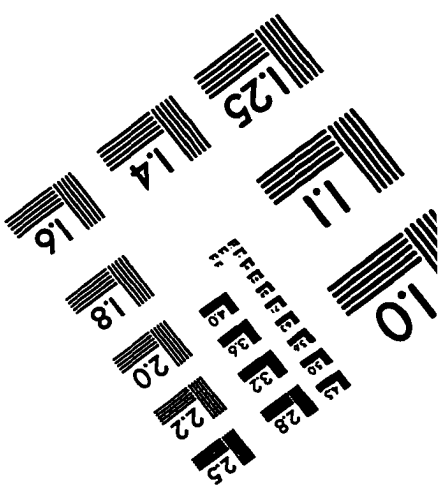












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