DETECTION OF K-RAS POINT MUTATION IN CODON 12 FOR
COLORECTAL CANCER BY THE ENZYME-LINKED
MINI-SEQUENCE ASSAY IN COMPARISON WITH
THE DOT-BLOT HYBRIDIZATION

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Key Words: Colorectal cancer, enzyme linked mini-sequence assay (ELMA), K-ras.
Subjects: Patients.
Abbreviations: DBH = Dot blot hybridization, ELMA = enzyme-linked mini-sequence
assay.

Abstract

The point mutation of K-ras codon 12 in the paraffin-embedded blocks of
colorectal cancer from 19 patients was analyzed with a newly developed detection
technique, enzyme linked mini-sequence assay (ELMA). The results were compared with
those obtained using conventional dot-blot hybridization (DBH) to examine the
consistency between these two techniques and the usefulness of the ELMA method. The
results of DBH showed that the point mutation of K-ras was present in 12 of 19 patients
(63.2%), while ELMA in the first trial showed it in 14 of 19 patients (73.7%). A
comparison of the results of DBH with those of ELMA revealed that the presence of the
mutation and its arrangement of base patterns agreed in 14 of the 19 patients (73.7%).
Then ELMA was repeated for the five patients in whom the results of the two techniques
did not agree. It showed that the second ELMA provided the results consistent with those
of DBH in four patients, but not in one patient. In three of these four patients, the color
intensity of mutation for the first trial of ELMA was (1+). However, the comparison with
DBH showed that the results agreed in 90% of the patients judged as (3+) by the first trial
of ELMA. The mutations examined by DBH and ELMA and the reproducibility of
ELMA agreed in 18 of 19 cases (94.7%) in accordance with the color intensity of 1(+) which
should be judged as a negative mutation. We conclude that these results confirmed
that ELMA for paraffin-embedded sections was useful in detecting the point mutation of
the K-ras gene.

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Introduction

The point mutation of K-ras gene is frequently observed in patients with pancreatic and colorectal cancers (Almoguera, Shibata et al., 1988; Bos, Fearon et al., 1987), and plays an important role in their carcinogenesis. However, its frequency of detection varies with the analytical technique used (Suchy, Zietz 1992; Sidransky, Tokino et al., 1992; Russo, Migliavacca et al., 1998; Aoki, Takeda et al., 1994). In this study, the point mutation of K-ras codon 12 in the paraffin-embedded blocks of colorectal cancer was analyzed with a newly developed detection technique, enzyme linked mini-sequence assay (ELMA, Fujino, Yatsuhashi et al., 1998). The results are compared with those obtained with conventional dot-blot hybridization (DBH) to examine the consistency between these two techniques and the eligibility of ELMA method.

Materials and Methods

Among those who received the surgical resection of colorectal cancer in the Tokyo Medical University Hospital, 19 patients examined by DBH for the point mutation of K-ras codon 12 were analyzed by the ELMA Method which has been established as SMITEST K-ras codon 12 GenotypeTM (Sumitomo Metal Industries Ltd, Tokyo, Japan) in Table I. Dot-blot hybridization: For the competitive dot-blot hybridization, cancer tissue samples resected and frozen during operation were used according to the method developed by Kawaguchi, Higashimoto et al. (1990). The region containing codon 12 was amplified by PCR using a thermal cycler. After the PCR products were fixed on a nylon membrane by dot-blotting, hybridization was performed using normal and mutation-typed allele-specific oligonucleotides as probes. As a result, a difference in one base was detected.

Extraction of DNA and PCR for ELMA

In order to prepare hematoxylin-eosin samples, a 6 μm-wide section was cut out from each formalin-embedded paraffin block at the region almost next to the DBH section. Cancer tissue was scraped from the sample under stereoscopy, and DNA was extracted with DEXPAT™ (Takara DNA Extraction from paraffin-embedded tissue, Kyoto, Japan). Oligonucleotide primers for K-ras exon 1 were artificially synthesized using a DNA synthesizer Model 392 (Applied Biosystems, Foster City, CA, USA). The
pairs consisted of upstream primer F1 (1st and 2nd PCR, 5'-TAAACTTGTGGTAGTGGAACT), downstreamer primer R1 (1st PCR, 5'-GTTGGATCATATTCGTACAC), and downstreamer primer R2 (2nd PCR, 5'-CAAATGATCTGAATTAGCTG). The 1st PCR amplifications were carried out with 25 µL reaction mixtures containing 1 µL (50 ng/µL) of template, 100 µM (each) deoxyribonucleotide triphosphates (dNTPs: dATP, dCTP, dGTP and dTTP), 1.5 mM MgCl2, 1 µM (each) primers F1 and R1, 0.625 U of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA), and 1x PCR buffer (10 x PCR buffer consisted of 100 mM Tris-HCl [pH 8.3 at 25° C], 500 mM KCl and 0.01% [w/v] gelatin). The reactions were performed in a PJ 2000 DNA thermal-cycler (Perkin-Elmer, Norwalk, CT, USA) with a mineral oil overlay. PCR amplifications proceeded at 95° C for 2 minutes followed by 25 cycles at 95° C for 40 seconds, at 60° C for 40 seconds and at 72° C for 40 seconds and a final extraction step of seven minutes at 72° C. 1 µL each of 10-fold dilutions of 1st PCR-amplified product (93 bp) were taken for digestions with 0.5 µL (2.5 U) Bsr I (New England Biolabs. Inc., MA, USA) and the 3.5 µL reaction buffer was composed of 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2 and 1 mM DTT at 65° C for more than 15 hours in a total of 5 µL. For digestion, 45 µL of the 2nd stage reaction mixtures containing 1 µM (each) primers F1 and R2, and the same other components as the 1st stage reaction mixtures were added to the above restriction endonuclease reaction tubes. Subsequently, the 2nd PCR amplifications were performed for 40 cycles with the same thermal cycle condition as the 1st PCR. The 1st PCR amplification of K-ras codon 12 with a mutation, which were not created at the cleavage site, and therefore, were amplified by the 2nd PCR again.

ELMA Method

Oligonucleotide probes for detecting the wild type and six-point mutations which adjusted the last three bases of the 3' position to each complementary sequence of K-ras gene codon 12 were immobilized on the microwell 6-plated as GAT, GCT, GGT, AGT, CGT, and TGT, respectively, in Figure 1. 10 µL aliquots of denatured 2nd PCR product were added to the microwell plate. The plate was gently tapped, covered and incubated for 30 minutes at 55° C. After five washes with 1 x wash buffer, the plate was tapped dry, and 100 µL of biotinylated A and Taq DNA polymerase were added. Then the plate was covered and incubated for 30 minutes at 55° C. After five washes, 100 µL
of avidin-horseradish peroxidase conjugate was added and incubated for 30 minutes at room temperature. After five washes, 100 μL of tetramethylbenzidine substrate was added. The plate was incubated in the dark for 20 minutes at room temperature. Finally, 100 μL of stop solution was added, and A-450s (absorbance in wave-length of 450 nm) were read with blanking using a spectrophotometer (Multiskan Multisoft, Labsystems, Finland).

The Criteria of Mutation

The mutation patterns of the samples were determined according to the following criteria: 1) Determination criteria for color developing intensity: When the absorbance of the normal GGT was less than 0.5, its mutated types were judged as negative. When their absorbance was less than 0.2, as intermediate when it was 0.2 or higher and less than 0.5, and as positive when it was higher than 0.5. When the absorbance of the normal GGT was higher than 0.5, its mutated types of GAT and AGT were judged as negative when their absorbance was less than 0.5, as an intermediate it was higher than 0.5 and less than 0.8, and positive when it was higher than 0.8. For the remaining four mutated types (GCT, GTT, CGT and TGT), the same criteria as the case where the absorbency of the normal GGT was less than 0.5 was used. 2) Determination criteria for mutation: (1) When only the normal GGT was positive (higher than 0.5) and all the others were negative or as intermediate, mutation was judged as (-). (2) When the normal and mutated types were positive and the absorbency of the mutated types was less than that of the normal GGT, mutation was judged as (1+). (3) When the normal and mutated types were positive and the absorbency of the mutated types was higher than that of the normal GGT, mutation was judged as (2+). (4) When only the mutated sequences were positive, mutation was considered as (3+). 3) The approximate ratio of the normal type to its mutated types: Based on the result of the mixture test of K-ras mutation positive cell stocks and human lymphoblast stocks, the ratio of the normal GGT to its mutated types of 1:5 or higher was considered (3+); that of 1/5 to 1/50 was (2+); that of 1/50 to 1/500 was (1+), that less than 1/500 was (-). When the results of DBH and ELMA did not agree, ELMA was tried again.
Results

The DBH showed that the point mutation of \textit{K-ras} codon 12 was present in 12 of 19 patients (63.2%), while ELMA in the first trial showed it in 14 of 19 patients (73.7%) which consisted of four cases of AGT mutation, four of GTT, four of GAT and two of TGT (Table I). (Figure 2).

### TABLE I

Patient characteristics and results of \textit{K-ras}

<table>
<thead>
<tr>
<th>No</th>
<th>Age</th>
<th>Sex</th>
<th>Duke’s Classification</th>
<th>Type of Histology</th>
<th>Dot-blot hybridization</th>
<th>ELMA (first trial)</th>
<th>ELMA (second trial)</th>
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<tr>
<td>1</td>
<td>50</td>
<td>F</td>
<td>C</td>
<td>Mod</td>
<td>GAT</td>
<td>(-)</td>
<td>GAT (2+)</td>
</tr>
<tr>
<td>2</td>
<td>63</td>
<td>M</td>
<td>B</td>
<td>Mod</td>
<td>AGT (1+)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>3</td>
<td>73</td>
<td>F</td>
<td>B</td>
<td>Mod</td>
<td>GTT</td>
<td>GTT (3+)</td>
<td>(-)</td>
</tr>
<tr>
<td>4</td>
<td>56</td>
<td>M</td>
<td>B</td>
<td>Mod</td>
<td>AGT (1+)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>5</td>
<td>67</td>
<td>F</td>
<td>A</td>
<td>Well</td>
<td>GTT</td>
<td>GTT (3+)</td>
<td>(-)</td>
</tr>
<tr>
<td>6</td>
<td>77</td>
<td>M</td>
<td>D</td>
<td>Mod</td>
<td>TGT</td>
<td>TGT (3+)</td>
<td>(-)</td>
</tr>
<tr>
<td>7</td>
<td>68</td>
<td>F</td>
<td>A</td>
<td>Well</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>8</td>
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<td>Well</td>
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<td>B</td>
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<td>58</td>
<td>M</td>
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<td>F</td>
<td>D</td>
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<td>GAT</td>
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<td>(-)</td>
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<tr>
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<td>Mod</td>
<td>GAT</td>
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</tr>
<tr>
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<td>74</td>
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<td>A</td>
<td>Well</td>
<td>TGT</td>
<td>TGT (3+)</td>
<td>(-)</td>
</tr>
<tr>
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<td>C</td>
<td>Mod</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>19</td>
<td>60</td>
<td>M</td>
<td>B</td>
<td>Well</td>
<td>(-)</td>
<td>AGT (1+)</td>
<td>(-)</td>
</tr>
</tbody>
</table>

Well: well-differentiated tubular adenocarcinoma; mod: moderately-differentiated tubular adenocarcinoma; muc: mucinous adenocarcinoma

A comparison of the results of DBH with those of ELMA revealed that the presence of a mutation and its arrangement of base patterns agreed in 14 of the 19 patients (73.7%). Therefore, ELMA was repeated for the five patients in whom the results of the two techniques did not agree in Cases 1, 2, 4, 14 and 19. It showed that the results of the second trial of ELMA were consistent with those of DBH in four patients (Cases 1, 2, 4 and 19), but not in one patient (Case 14). In three of the four patients (Cases 2, 4, and 19), the color intensity of mutation for the first trial of ELMA was (1+).
When the order of codon 12 in 2nd PCR product is \textit{GAT}, color comes out by the marked substance \textit{A}.

Color does not come out by the absence of the marked substance \textit{A} due to the mismatch of the order of codon 12.

\textit{Figure 1:} Reaction theory of ELMA method.
In Case 1, the color intensity of mutation was negative in the first ELMA, but (2+) in the second trial. The results for ten patients who obtained the (3+) of color developing intensity in the first ELMA were also positive and consistent with those of DBH. All of them except for Case 14 (90%) showed the same mutation patterns between DBH and ELMA. The arrangement of base patterns by DBH in Case 14 was GCT, while it was GAT (3+) by the first and second trials of ELMA.

**Case No.**

![Figure 2: Actual image of microwell in which color came out on each mutated plate.](image)

**Discussion**

*K-ras* is one of the cancer genes located in the short arm of the 12th chromosome, and it is frequently detected in codon 12. Various techniques have been developed to detect the *K-ras* gene since the 1980's, including dot-blot hybridization (Suchy, Zietz *et al.*, 1992), restriction fragment length polymorphism (Sidransky, Tokino *et al.*, 1992),
single strand conformation polymorphism (Russo, Migliavacca et al., 1992), mutant allele-specific amplification (Aoki, Takeda et al., 1994) etc. However, these conventional techniques have pointed out some problems of specificity, reproducibility and complicated management, which are therefore inadequate for the ordinary clinical examination (Fujino, Yatsuhashi et al., 1998). On the other hand, the ELMA method has several advantages. It is technically easier because it does not use a radio immunoassay, it does not require electrophoresis and it is not needed to confirm the types of mutation by direct sequence. ELMA can detect multiple mutation patterns at the same time, which means that it does not need to take a long time in comparison with conventional techniques. Finally, it is stable and shows a high reproducibility which has been already proven for detection of the HBV-DNA pre-core mutant (Fujino, Yatsuhashi et al., 1998).

From these points of view, the ELMA method is thought to be very adequate for the routine work in the clinical investigation of the comparison with conventional methods.

In this study, DNA was extracted from formalin-fixed and paraffin-embedded blocks. In order to analyze DNA, an extract from fresh samples is more desirable because it can avoid the damage by formalin. However, the extraction of DNA from paraffin blocks will become increasingly necessary because it is needed to examine the past few valuable cases and to allow the collection of target cells. These target cells are morphologically coincident when seen under the stereoscope and can, therefore, prevent contamination.

A comparison with DBH showed that the results agreed within 90% of the patients judged as (3+) by the first trial of ELMA, indicating the clinical usefulness of ELMA. The three patients judged as (1+) in cases 2, 4 and 19 showed a low color developing intensity, and they were judged as (-) in the second ELMA. In general, ensuring sufficient reproducibility for repetitive measurements is more difficult when there is DNA extraction from a small amount of cancer tissue in the paraffin section as a template of PCR amplification in comparison with that using fresh tissues because of a low DNA extraction efficiency in the change of DNA to lower molecules in pathologic fixation and the effects of contaminants that inhibit PCR reaction. The results of the present study indicate that it would be appropriate to judge the three patients in whom the (1+) signal could not be reproduced as negative. In addition, when the ratio of the normal
type to its mutated types of (1+) has been observed in spite of the fact that only cancer tissue was selectively scraped from a paraffin section. Its cellularity might be inconsistent and re-examination should be necessary.

Moreover, the mutation patterns by DBH and ELMA were different in Case 14. As reported by various investigators (Anker, Lefort et al., 1997; Hayashi, Egami et al., 1997; Takenaka, Uehara et al., 1997), multiple mutation patterns have been observed at the same site in pancreatic and colorectal cancers. Therefore, it would be appropriate to evaluate Case 14 as expressing multiple mutations. However, strictly speaking, it would be necessary to re-examine the dot-blot hybridization and/or to compare the mutation rate of sub-cloning from the PCR products in order to distinguish mis-hybridization from the presence of a small amount of mutation clone, when the results are inconsistent between both the methods. In Case 1, the mutation was negative in the first trial of ELMA, but it showed GAT 2(+) in the second trial. This was the only case where (2+) could be obtained by ELMA. However, DBH also showed the same result. The (2+) could be considered positive for mutation.

In summary, the mutations examined by DBH and ELMA and the reproducibility of ELMA agreed in 18 of 19 cases (94.7%) in accordance that the color intensity of 1(+) should be judged as a negative mutation. These results confirmed that ELMA for paraffin-embedded sections was useful for detecting the point mutation of the K-ras gene.

References


