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Utilization of *K-ras* Mutations Identified in Stool DNA for the Early Detection of Colorectal Cancer

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Abstract Colorectal cancer is one of the most common malignancies in the western world. About 60,000 Americans die of colorectal cancer each year. The annual incidence rate in Israel is 40 per 100,000 persons, namely a total of 2,000 new cases each year. An important step in the progression of colorectal cancer includes induction of activating mutations in the proto-oncogene *K-ras*. The mutations in *K-ras* appear early during tumorigenesis, at the intermediate adenoma stage, and thus can be used as a biomarker for early detection in about 40% of colonic tumors. A large yet unknown number of mutated cells are shed from the developing tumor during its progression. Indeed, *K-ras* mutations were detected in DNA isolated from stool obtained from symptomatic and asymptomatic patients with colorectal cancer, suggesting a novel approach for a noninvasive screening procedure. However, severe difficulties in obtaining reproducible yields of amplifiable DNA from stool, and usage of nonquantitative, time-consuming procedures, hampered further progress in the utilization of *K-ras* mutations for the early detection of colorectal cancer. Apparently a novel protocol is required that provides reproducible output of amplifiable DNA from small amounts of stool, detects if *K-ras* mutated DNA is present, and determines the quantity of *K-ras* mutated cells in the stool sample. In addition, this protocol should be simple, robotics compatible, and thus suitable for cost-effective, large-scale mutation screening. Molecular assays for detecting *K-ras* mutations and additional biomarkers in stool DNA promise to be highly sensitive, specific, and cost-effective. As such they should be very effective when used in chemoprevention studies and screening protocols for colorectal cancer. J. Cell. Biochem. Suppl. 34:35–39, 2000.

Key words: cancer genetics; sporadic tumors; mutation screening; DNA isolation; automation

Incidence and Diagnosis of Colon Cancer

Colorectal cancer is one of the most frequent malignancies in the western world. In Israel it is the most common cancer among men, and the second most common among women. The annual incidence rate is 40 per 100,000 persons, namely a total of 2,000 new cases each year. In the United States, where colorectal cancer is the second most common cause of cancer death (after lung cancer), about 60,000 Americans die of this malignancy each year [McMichael, Giles 1994]. Fortunately, colorectal cancer can be cured by relatively simple surgical procedures. Thus, while mortality from advanced disease is frequent or even imminent (Duke's C and D, 5-year survival rate of 40% and 0%, respectively), it can be efficiently prevented by early detection (Duke's A and B, 5-year survival rate of 90% and 70%, respectively). The incidence of colorectal cancer rises sharply in elder people, and it is generally accepted that persons over 50 years of age should be checked routinely for colorectal cancer, at least once every 3–5 years. In the western world about one-third of the population is in this category, suggesting that, in the US, 20 million tests are required annually. The corresponding figure in Israel is 500,000.

The common protocols for early detection of colorectal cancer, including colonoscopy, flexible sigmoidoscopy, barium enema testing, or hydrocolonic sonography; all involve uncomfortable, invasive procedures. In addition, some of these approaches are not optimal for screening due to their high cost. Screening for colon cancer by fecal occult blood testing (FOBT) is con-

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sidered cost-effective but suffers from limited sensitivity and specificity. In two studies, a significant reduction in mortality was shown [Winawer et al., 1993; Mandel et al., 1993], but in others, no influence on mortality was proved [Ahlquist et al., 1993; Kronborg et al., 1996]. Thus, although a deliberate combination of established screening protocols (such as endoscopy, flexible sigmoidoscopy, and FOBT) could reduce mortality by 30%, the need for more sensitive, specific, noninvasive, and cost-effective screening protocols is apparent.

K-ras as An Early Molecular Marker for Colorectal Cancer

Less than 10% of colorectal tumors are due to hereditary syndromes such as adenomatous polyposis coli (APC) or hereditary nonpolyposis colon cancer (HNPCC), and 90% originate from nonhereditary, sporadic colon cancer. This somatic, nonfamilial neoplasm became a model for multistep cancer progression based on the elucidation of genetic changes that accumulate during histopathologic progression [Fearon and Vogelstein 1990; Kinzler and Vogelstein 1996]. Apparently, early steps in the progression from normal gut epithelium to benign adenoma include inactivation of *APC* and activation of *K-ras*, and the transition from adenoma to carcinoma is caused by inactivation of *DCC* and *p53*. Consequently, mutations in *APC*, *p53*, and *K-ras* are highly frequent in colorectal tumors, suggesting their potential for use as molecular diagnostic markers [Sidransky, 1997]. Since even small adenomas almost ubiquitously contain *APC* mutations, and since most adenomas (>90%) do not progress to malignancy, the use of *APC* mutations as a molecular marker may be impractical. Mutations in *p53* appear in most colonic tumors (~70%), but mainly in carcinomas already in the advanced malignant stage. Consequently mutations in *p53* are not relevant for early detection.

Mutation in *K-ras*, on the other hand, are somewhat less frequent, appearing in about 30–50% of colorectal tumors. However, they are confined to only two sites, namely codons 12 and 13 of *K-ras*. Mutations in codon 61 are very rare in colorectal tumors. For example, in Israeli patients, no codon 61 mutations were found in the first 30 colorectal carcinomas tested [Z. Lev and D. Kislitsin, unpublished results]. Furthermore, the *K-ras* mutations in codons 12 and 13 are detectable as early as the benign adenoma stage [McLellan et al., 1993], and they are found more frequently in relatively large adenomas, more than 1 cm in diameter [Morris et al., 1996]. This later finding is prognostically important since larger adenomas have a greater chance for progression than smaller ones [Fearon and Vogelstein, 1990]. In malignant colorectal tumors, *K-ras* mutations are found with similar frequency to mutations in intermediate adenomas. Hence, the preferable time of *K-ras* mutation occurrence is the intermediate adenoma stage, a stage that is considered as already clinically dangerous.

Detection of *K-ras* Mutations in Stool DNA

It all started with the pioneering work of Sidransky and colleagues [1992] who were the first to identify mutated *K-ras* DNA in stool samples of colorectal cancer patients. In eight out of nine cases where a mutation was found in the tumor, an identical one was found in the stool DNA, strongly suggesting that the mutated stool DNA originated from cells shed by the tumor into the gut. In addition, this study included not only carcinomas but also two adenomas, with one of them as small as 1 cm^3. Surprisingly, the percentage of mutated alleles was relatively high, at least in the amplified DNA used for the detection, reaching 4–20% of total *K-ras* alleles. It should be noted that the tumor contains a large number of cells even when compared with the gut epithelium, and the DNA in its cells may be less prone to apoptotic degradation [Sidransky et al., 1992]. Another source for these cells is the mucose surrounding the tumor, which is also very rich in tumorous cells [Z. Lev and D. Kislitsin, unpublished results]. In fact, it is much easier to get amplifiable DNA from stool samples obtained from cancer and inflammatory bowel disease (IBD) patients than from healthy persons [Villa et al., 1996; Loktionov et al., 1998].

It is clear that this study opened the door to a possible novel, noninvasive screening protocol for the early detection of colorectal cancer. A subsequent study went one step further, as *K-ras* mutations were looked for in 39 patients at risk for colorectal cancer but without apparent clinical symptoms [Tobi et al., 1994]. Colonic effluent samples were taken prior to routine colonoscopy and 18% of them were positive for *K-ras* mutation. One of these patients indeed developed a tumor 4 years later. In this study a very sensitive method was introduced,

termed enriched PCR [Kahn et al., 1991], which could detect *K-ras* mutated allele diluted more than 10,000-fold with normal alleles. Enriched PCR could detect *K-ras* mutations even in normally appearing mucosa of colorectal cancer patients [Minamoto et al., 1995]. Additional studies [Smith-Ravin et al., 1995; Hasegawa et al., 1995; Koornstra et al., 1995; Ratto et al., 1996; Nollau et al., 1996] including a most comprehensive one that was carried out with 230 patients [Villa et al., 1996], confirmed the high sensitivity and specificity of the *K-ras* mutation assay. For example, very low (<10%) false negatives were obtained, and essentially zero false positives. In other words, more than 90% of *K-ras* mutated tumors were detected by testing the corresponding stool DNA, and no *K-ras* mutations were found in stool of healthy, not-at-risk persons. In parallel, *K-ras* mutations were detected in stool of patients with pancreatic adenocarcinoma [Caldas et al., 1994; Berndt et al., 1998], and in lavage fluid of patients with ulcerative colitis [Lang et al., 1997].

How Long We Will Have to Wait?

Several features and characteristics of *K-ras* mutations in colorectal tumors suggest that these mutations could be exploited as an excellent tool in colon cancer detection and management: a) *K-ras* mutations appear in a large fraction of non-familial, sporadic tumors; b) they appear early during tumorigenesis, but not too early, and thus can be used for early detection; c) the mutations are just a few, clustered in two codons only; d) a large number of mutated cells are shed from the developing tumor and are detected in stool obtained from symptomatic and asymptomatic patients, thus a noninvasive screening procedure could be used; e) molecular methods for mutation detection are sensitive and specific. In view of these apparent advantages of *K-ras* mutations for the early detection of colorectal cancer, they should have already been exploited in routine screenings of populations at risk such as first-degree relatives of patients with colorectal cancer, patients with ulcerative colitis, Crohn's disease patients who tested positively in the fecal occult blood test (FOBT), and people aged 60 and older. It is very discouraging to point out that this is not the case [Sidransky, 1994; Villa, 1997]. So, how long we will have to wait for the implementation of a screening plan based on the detection of *K-ras* mutations? A closer look at the number of studies that approached this challenge suggests that further progress has been hampered by severe difficulties in obtaining reproducible yields of amplifiable DNA from stool. Thus, in one study which included over 200 cases, only 45% of the amplifications were successful [Villa et al., 1996], and in another one only 51% of 35 samples were amplifiable [Potter et al., 1997]. In addition, all these studies were carried out by time-consuming methods including enzymatic reactions, gel-electrophoresis, or filter hybridization assays. Consequently, in most cases only a small number of samples were tested. Finally, current assays are not quantitative. Thus, they cannot distinguish between cells shed from a relatively large tumor, and cells originating in small microadenomas or other fortuitous sources for *K-ras* mutated cells.

The ideal protocol should overcome all these difficulties [Jen et al., 1998]. It must provide reproducible output of amplifiable DNA from small amounts of stool. In addition, it should be based on a quantitative assay for the amount of *K-ras* mutated cells in stool samples, thus enhancing its potential as a prognostic assay. Finally, it should be robotics compatible, and thus suitable for cost-effective, large-scale mutation screening. Examples for efforts in this direction are a simplified oligonucleotide ligation assay [Rothschild et al., 1997], a quantitative enriched PCR method [Ronai and Minamoto 1997], and a microplate assay for *K-ras* genotyping [Berndt et al., 1996].

Concluding Remarks

In the long run, improved molecular assays based on *K-ras* mutations promise to be highly sensitive, specific, and cost-effective. Although only about 40% of tumors are expected to be identified using this marker, it should be noted that genetic markers for inherited colorectal cancer such as *APC* and *HNPCC* are able to identify no more than 10% of the potential patients. Compared with other common sporadic cancers such as lung or breast cancers, this figure is unsurpassed by any other markers used for early detection of the three major cancers. In any case, it is expected that, in the near future, additional biomarkers will be tried in this system. These could be genes already known to be genetically altered in colorectal cancer such as *p53, APC, DCC* [Kinzler and Vogelstein, 1996]. Another promising source for

novel biomarkers are polymorphic microsatellites, recently found to be associated with other neoplastic tissues [Mao et al., 1994; Sidransky, 1997]. Finally, differential analyses of gene expression patterns specific to cancerous cells by novel molecular approaches such as the SAGE and DNA microarray systems [Zhang et al., 1997; DeRisi et al., 1996] will certainly provide a variety of new tools amenable for early detection of colorectal cancer. Since the chances of curing colorectal cancer are significantly improved by early diagnosis, a successful utilization of *K-ras* mutation detection in stool DNA would pave the way for the utilization of a broader spectrum of biomarkers, helping to reduce morbidity and mortality caused by this major malignancy.

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