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Screening for Clonal Genetic Alterations

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Clonality is a fundamental characteristic of all human cancers. One cancer cell gives rise to daughter cells, all of which exhibit the same change that initially provided a growth advantage to the parent cell. Accumulation of further genetic changes in subsequent daughter cells, each providing an additional growth advantage, has been well documented in human cancer. Correlation of these clonal genetic changes with histopathological progression has led to development of a molecular progression model for colorectal cancer. The identification of genetic markers able to identify the clonal outgrowth of neoplastic cells has proven useful in the detection of a variety of primary neoplasms.

Key words: molecular screening, cancer detection, genetic alterations
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CLONAL OUTGROWTH is a common feature of all human neoplasms [1, 2]. This important observation was confirmed by molecular approaches including X-chromosome inactivation, where neoplasms were found to be derived from a single cell that had inactivated either the maternal or paternal X-chromosome [3–5]. The subsequent clonal outgrowth of more aggressive clones was recognised as an important clinical phenomenon leading to the invasion and progression of human neoplasms [2, 6, 7]. The histopathological progression of colorectal cancer, commonly known as the adenoma to carcinoma sequence, now serves as a paradigm for the progression of most human cancers. Classic work by Vogelstein and colleagues demonstrated that these histopathological changes are driven by inactivation of proto-oncogenes and inactivation of tumour suppressor genes [8]. These genetic changes correlate with the various stages of colorectal cancer progression, and have now been placed in a general molecular progression model. From this progression model, it is clear that certain genetic changes such as *KRAS* activation or inactivation of the *APC* (adenomatous polyposis coli) gene occur early in progression [9]. Alternatively, loss of 17p associated with inactivation of the *TP53* gene [10] and loss of 18q associated with inactivation of *DCC* (deleted colon cancer) gene generally occur late in the progression pathway. Moreover, multiple areas of chromosomal deletions have been identified, but not yet characterised along this molecular progression model. Genetic changes that occur early in progression may take place in preneoplastic lesions that are still in an asymptomatic stage of clinical development. We initially reasoned that these genetic changes may provide rational markers for early cancer diagnosis.

We have demonstrated that point mutations in critical oncogenes and tumour suppressor genes can be used in PCR (polymerase chain reaction) detection of occult neoplastic cells in bodily fluids [11, 12]. Our initial target in colorectal cancer was the *RAS* proto-oncogene which occurs in approximately 30–50% of both adenomas and carcinomas. The *RAS* proto-oncogene was a particularly attractive target because mutations were clustered within codons 12 and 13, thus facilitating the technical detection of only a few point mutations. In our first colorectal study, we accumulated 12 cases of primary colorectal carcinomas and corresponding stool samples from affected patients. We isolated and purified DNA from these stool samples, and then tested these samples for the presence of clonal *RAS* gene mutations.

Our initial approach was based on PCR, followed by cloning and plaque hybridisation. PCR allows amplification of DNA a million-fold to allow assessment of minute quantities of DNA. The plaque hybridisation assay employed cloning of these PCR products into bacteriophage, so that each individual plaque on a plate would represent one *RAS* allele. The plaques were then transferred to nylon membranes and hybridised with specific probes able to recognise the different codon 12 and 13 mutations of *RAS* often present in primary tumours. This technique has the ability to detect one cancer cell among a background 10 000 normal cells [11, 12].

8 of the 9 patients with a *RAS* proto-oncogene mutation in a primary colorectal cancer demonstrated positive hybridisation by this plaque assay in the corresponding stool sample. A single patient with a *RAS* gene mutation and a deep rectal neoplasm was negative by our assay. Importantly, the remaining three patients without *RAS* oncogene mutations in the primary tumours and the three controls without cancer were completely negative by this approach. Moreover, patients with primary tumours far in the ascending colon and patients with early adenomas were also detected by this assay.

Since then, others have used molecular approaches to detect *RAS* gene mutations in the stool of pancreatic cancer patients (Table 1). Some of these studies employed simpler assays that did not necessitate cloning, such as allele-specific amplification and "enriched" PCR, all able to successfully detect rare *RAS* gene mutations in various bodily fluids [13, 14]. In all these studies, the detection of *RAS* gene mutations has corresponded with identification of the same *RAS* gene mutations present in

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Table 1. Molecular screening studies in primary cancers

Study	Neoplasm	PCR-based technique	Clinical sample
Sidransky *et al.*, 1991 [11]	Bladder	Plaque hybridisation	Urine
Sidransky *et al.*, 1992 [12]	Colon	Plaque hybridisation	Stool
Tada *et al.*, 1994 [14]	Pancreas	Allele-specific amplification	Pancreatic juice, blood
Caldas *et al.*, 1994 [13]	Pancreas/bile duct	Plaque hybridisation	Stool
Tobi *et al.*, 1994 [15]	Colon	"Enriched" PCR	Colonic effluent
Mao *et al.*, 1994 [23]	Lung	Plaque hybridisation	Sputum
Mao *et al.*, 1994 [21]	Bladder, lung	Gel electrophoresis	Urine, sputum

The details of each of the individual molecular techniques are described in the respective studies. All but the last study (microsatellite alterations) involve detection of proto-oncogene or tumour suppressor gene mutations in the clinical samples.

the primary tumours. In some cases, where there was a second *RAS* gene mutation present in stool, careful microdissection and sequence analysis of the primary neoplasm revealed the presence of a second competing *RAS* gene mutation in the primary pancreatic cancer [13]. These studies have now confirmed and expanded our initial results, and demonstrate the power of novel molecular approaches.

A recent study was noteworthy in that asymptomatic high risk patients with a personal or family history of colon cancer were tested [15]. Although these patients did not have clinical symptoms or visible tumours, investigators demonstrated clonal *RAS* mutations in a high percentage of corresponding colonic effluent samples. It will be of great interest to continue to follow-up these patients for the development of pre-invasive or invasive colorectal cancers. This approach demonstrates the potential use of molecular screening for early diagnosis, since gene mutations were detected before patients went on to develop cancer. Moreover, false positives have been quite rare in these molecular studies, and it will be of great interest to see if this type of specificity can be maintained in larger clinical trials.

One major limitation of these molecular screening approaches has been the necessity to identify many different mutations in a variety of oncogenes. In broad terms for general cancer screening, molecular progression models vary significantly from one tumour type to another. Although the progression model for colorectal cancer is by far the best delineated, the ideal genetic target is not clear. Certainly, only a minority of tumours will have a mutation of the *RAS* oncogene, and identification of *APC* mutations is technically very difficult, due to the large size of the gene and the occurrence of many mutations in primary tumours. It would be necessary to use hundreds of different probes to identify the many types of *APC* mutations by a plaque hybridisation assay. Similarly, multiple specific primers would have to be used for allele-specific amplification strategies. However, the recent identification of microsatellite instability in hereditary nonpolyposis coli (HNPCC) has suggested a new opportunity for molecular detection [16–18]. Primary tumours from these affected Lynch syndrome cancer patients have demonstrated diffuse genomic instability, manifested as deletions or expansions of small repeat units throughout the human genome. Current evidence suggests that these alterations probably arise in transformed cells as a replication error during cell division. They are then propagated to daughter cells (harbouring a shared genetic event that provides a growth advantage) during clonal expansion. Primary tumours and cell lines derived from these patients suggest that they have deficiencies in one or more of several mismatch repair genes involved in DNA repair [19, 20].

The detection of these novel expansions or deletions appears restricted to monoclonal neoplastic tissue. A replication error in a cell that does not undergo clonal expansion should not be detected among the large excesses of normal DNA from surrounding cells. Therefore, a single PCR amplification of DNA from clinical samples allows detection of these clonal genetic alterations by simple gel electrophoresis. Microsatellite alterations are visualised as aberrantly migrating bands when compared to the migration pattern of normal DNA (e.g. blood) from the patient. We have demonstrated the presence of these clonal microsatellite alterations in urine samples from patients with bladder cancer, and sputum samples from patients with lung cancer [21]. We have also identified certain (hypermutable) microsatellite loci that might be more susceptible to these expansions or deletions. These alterations often occur in larger (three or four base) repeats that still occur commonly throughout the human genome. Of interest, some markers appear to be altered in a tissue-specific pattern; certain tumours demonstrate frequent alterations of one marker, while other tumours demonstrate higher alterations in another microsatellite repeat.

Colorectal cancers could be particularly amenable to this detection strategy because of the high frequency of microsatellite alterations observed in sporadic tumours. Development of this type of detection strategy will require extensive testing of both small and large repeat units in paired normal tumour samples to identify the best possible markers. In addition, we have demonstrated that multiplex assays can "pool" several of these markers in one PCR reaction to detect the largest number of primary tumours possible [21]. This method is less sensitive than some of the other assays listed previously. However, it can still detect one neoplastic cell among approximately 1000 normal cells, and is much more feasible requiring PCR amplification without additional cloning. Prospective evaluation of clinical samples by this assay should identify patients at high risk of neoplastic progression or those who have already developed cancer. Because of the technical ease required for this test, it may offer a relatively low cost molecular approach for cancer detection.

Clonal genetic alterations appear promising as molecular diagnostic tools for colorectal cancer screening. Assays that allow simplified detection of critical proto-oncogene and tumour suppressor gene alterations will make this type of screening more practical. Additionally, the use of microsatellite alterations may already allow a cost-effective means of screening. The accumulation of paired primary tumour and stool samples from affected patients for rapid validation of this technology has now become imperative [22]. As technology improves, these

approaches will need to be taken to the clinical setting for prospective trials with many patients. Although the effort and cost to run these trials may appear daunting, the promise of early detection and higher cure rates looms on the horizon. Success depends on cooperation between scientists and clinicians to continue to bring molecular biology from the bench to the bedside.

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