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APPLICATION OF DNA MARKERS TO CLINICAL GENETICS

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Summary DNA technology using DNA sequence polymorphisms has brought a new system to the fields of medicine and forensic science, especially for the studies of genetic diseases and tumor suppressor genes, and for identification of individuals for forensic purposes. Linkage analysis based on segregation of polymorphic alleles in affected families has contributed to identification of many genetic diseases. We isolated a large number of polymorphic DNA markers, called VNTR (variable number of tandem repeat) markers and identified the APC gene that is responsible for familial adenomatous polyposis (FAP) by means of a so-called "positional cloning" and characterized germline and somatic mutations of the APC gene in colorectal cancer patients. In addition, we have applied genetic information during colorectal carcinogenesis to sensitive diagnosis of lymph-node metastasis of colorectal cancer.

Key Words RFLP, VNTR, linkage analysis, positional cloning, APC

Polymorphic DNA markers

Segregation studies based on polymorphic DNA markers, normal variations in DNA at many sites in the human genome, often allow us to distinguish whether a chromosome is of maternal or paternal origin. When that is possible, we can follow the inheritance of a gene that is known only by a phenotype that is segregating in a family, if the phenotype co-segregates with a polymorphic "marker" locus. Co-segregation, or "linkage," depends on physical proximity on a chromosome between the marker and the unknown gene. Genetic linkage analysis using this powerful property of normal variations has been contributing to isolate genes responsible for human genetic diseases such as Duchenne muscular dystrophy, cystic fibrosis, polycystic kidney disease, and familial adenomatous polyposis.

Conventional polymorphic DNA loci consisted of only two possible alleles, generated by a substitution of one nucleotide for another in human genomic DNA,

at a site recognized for cleavage by a bacterial endonuclease ("restriction enzyme"). We developed another type of RFLP, known as a VNTR (Variable Number of Tandem Repeat) (Nakamura *et al.*, 1987, 1988a), the second-generation polymorphic DNA marker, that is generated by variation from one individual to another in the number of tandemly-repeated sequences at a particular locus. Because the number of different alleles possible in the population can be large at such a locus, VNTRs are very informative in a linkage study. VNTRs are also helpful in forensic studies because of their highly polymorphic nature (Odelberg *et al.*, 1989; Kasai *et al.*, 1990), and they are being used in clinical settings to monitor recipients of bone marrow transplants (Gatti *et al.*, 1989). However, in recent years, a huge number of the third-generation polymorphic DNA marker, microsatellite DNA markers were identified and have been used for linkage analysis (Gyapay *et al.*, 1994). The microsatellite markers are generated by variation in the number of short tandemly-repeated sequences like CA, CT, or CAA at a particular locus, and are amplified by the polymerase chain reaction.

Construction of chromosomal maps

For the construction of a genetic linkage map that will be useful in detecting the location of a genetic defect segregating in a family, we need to estimate the distance between DNA markers. Such estimates derive from the frequency of recombination events between pairs of markers. During meiosis, the homologous chromosomes line up in pairs; crossing-over of segments of DNA occurs between the homologs, and this exchange of material (recombination) produces chimeric chromosomes, a patchwork of DNA segments derived from each parent.

During one meiosis, an estimated 30 recombination events occur throughout the genome. From this phenomenon and the estimated size of the human genome (3×10^9 base pairs), we can calculate roughly that a 1% frequency of recombination between two markers reflects an average of 1,000 kb, or 1 million nucleotide base pairs, of physical distance on a chromosome. Our analysis of genotypic data for DNA markers in a large number of 3-generation families, using a sophisticated computer program, has produced genetic linkage maps for almost all human chromosomes (O'Connell *et al.*, 1989a, b, c; Lathrop *et al.*, 1988a, b, 1989; Nakamura *et al.*, 1988b, c, d, e, 1989a, b; White *et al.*, 1989; Julier *et al.*, 1990a, b; Yamakawa *et al.*, 1991a; NIH/CEPH Collaborative Mapping Group, 1992).

In addition to genetic linkage maps, we have constructed cytogenetic chromosomal maps using fluorescent *in situ* hybridization (FISH) technique. We constructed cosmid libraries from human-mouse somatic hybrid cell lines, that contained single human chromosome as the only human complement. By colony hybridization with labeled total human DNA as a probe, we selected chromosome-specific cosmid clones and constructed high-resolution cytogenetic maps with the average distance between markers of 250 kb–1 Mb. A total of more than 3,300 genomic cosmid markers have so far been mapped on chromosomes 1, 2, 3,

5, 6, 8, 9, 11, 12, and 17 (Yamakawa *et al.*, 1991b; Hori *et al.*, 1992; Takahashi *et al.*, 1992, 1993a, b, 1994a, b; Emi *et al.*, 1992; Inazawa *et al.*, 1993; Nakamura *et al.*, 1994; Ariyama *et al.*, 1995).

Mapping of the loci for tumor suppressor genes

To investigate the loci that contain tumor suppressor genes, we have examined a large number of sporadic carcinomas of the lung, esophagus, liver, ovary, breast, and kidney for loss of heterozygosity (LOH), using polymorphic DNA markers representing all autosomal chromosomes (Fujimori *et al.*, 1991; Morita *et al.*, 1991; Sato *et al.*, 1991a, b; Tsuchiya *et al.*, 1992; Aoki *et al.*, 1994). In esophageal squamous cell carcinomas, allelic losses at frequencies of at least 30% were observed at loci on chromosomal arms 3p (33%), 3q (30%), 5q (36%), 9p (57%), 9q (60%), 10p (33%), 13q (43%), 17p (62%), 17q (46%), 18q (38%), 19q (32%), and 21q (37%), suggesting that several putative tumor suppressor genes associated with development and/or progression of esophageal cancer on these chromosomal arms. By a comparison of LOH on each chromosomal arm with clinicopathological parameters of patients, we have found that the frequency of LOH on 17q was significantly higher in tumors in female patients (12 of 14 cases) than in those in male patients (20 of 56 cases) ($p=0.0009$ by Fisher's exact test). In hepatocellular carcinomas, we found that a putative tumor suppressor genes for HCC are likely to be located on chromosome 5q, 10q, 11p, 16q and 17p. In renal cell carcinoma (RCC), our results implicated a presence of tumor suppressor genes on seven chromosomal arms, chromosomes 3p, 5q, 6q, 10q, 11q, 17p, and 19p. In ovarian cancers, frequent LOHs were observed on chromosomes 4p, 6p, 7p, 8q, 12, 16, 17, and 19p.

Isolation of the gene responsible for familial adenomatous polyposis (FAP)

Familial adenomatous polyposis (FAP) is an autosomal-dominant disease, that affects 1 in 7,000–15,000 people worldwide. FAP is characterized by development of hundreds to thousands of adenomatous polyps in the colon and rectum, one or more of which will progress to cancer if left without surgical treatment. Cytogenetic observation in one FAP patient (Herrera *et al.*, 1986) and linkage studies in large FAP kindreds (Bodmer *et al.*, 1987; Leppert *et al.*, 1987; Nakamura *et al.*, 1988f) localized the gene responsible for FAP to chromosome 5q21-22.

By means of "positional cloning," we and others succeeded in identifying the gene responsible for FAP, termed the APC (adenomatous polyposis coli) gene, which codes 2843 amino acids (Kinzler *et al.*, 1991; Nishisho *et al.*, 1991; Groden *et al.*, 1991; Joslyn *et al.*, 1991). The APC gene is expressed ubiquitously in various organs, but at least five forms of non-coding transcripts have been identified (Horii *et al.*, 1993). We have investigated germline mutations in patients with FAP. The mutations of the APC gene was found in 70% of 150 FAP patients

that were screened the entire coding region by an RNase protection assay. Of the 106 APC germ-line mutations that we detected, 66 were 1-8 bp deletions and three were 1-2 bp insertions; all caused a shift of the APC reading frame, that created new stop codons downstream. Of 39 point mutations, 33 generated stop codons and six led to amino acid substitutions. One was intronic sequence deletion that could affect a splicing. Thus, 100 (94%) of the 106 mutations so far detected were predicted to result in truncation of the APC protein (Nagase and Nakamura, 1993; Nakamura, 1993). Only 6% of the mutations so far found were considered to induce substitution of an amino acid. These results have implied that detection of truncated APC protein by antibody may be an easy and simple method for presymptomatic diagnosis of individuals carrying APC mutations.

It is well known that deletions often occur where nucleotides are repeated, perhaps because of mis-alignment. Our observations, which included a C deletion from CCC (at codon 1427), an A deletion from several A's (codon 142) and an AA from AAA (codon 1250), were consistent with a proposed model for generating mis-alignment within a stretch of common bases. Some of other deletions in APC have occurred at positions containing multiple copies of a direct repeat; for example, a 2-base deletion from a sequence containing five AG repeats (AAAGAGAGAGAGTG; codon 1465), a 5-base (AAAGA) deletion at ATAAAAGAAAAGATT (codon 1309); a 5-base (ACAAA) deletion from ATAAAACAAAGT (codon 1061), and a TGAAA deletion from TCAAATGAAAAC (codon 1546) have been detected. All of these deletions may have occurred during DNA replication, probably as a result of slippage of the template strand and subsequent mis-alignment. After synthesis of the first copy of the direct repeat, the template strand could slip and mis-align with the second copy of the repeat, with the result that the intervening sequences would be deleted.

Comparisons of germ-line mutations with clinical manifestations other than colorectal polyposis, in 29 FAP patients for whom detailed information was available. Although FAP is frequently accompanied by extra-colonic manifestations, observations of variable phenotypes of patients within single pedigrees have suggested that extra-colonic manifestations are not regulated simply by the mutation causing a large number of polyps in colon and rectum. Our results support the view that extra-colonic manifestations, *i.e.* osteomas, epidermoid cyst, desmoid tumors, thyroid tumors, and upper gastrointestinal polyps do not correlate with the type or location of a particular germ-line mutation. We also compared the locations of germ-line APC mutations in 25 unrelated patients with the number of colorectal polyps developed in each case; 19 patients were sparse types and six were profuse types. All but one of the mutations were considered to cause truncation of the gene product by frame-shift, due to deletion or nonsense mutation (Nagase *et al.*, 1992a, b). The location of each germ-line mutation seemed to correlate with clinical type; mutations in the FAP patients with profuse polyps were observed between codons 1250 and 1464, whereas mutations in the

FAP patients with fewer polyps had occurred in other regions of the APC gene. The result suggests that the number of colorectal polyps in FAP patients may be associated with a difference in the stability or the biological function of the truncated APC protein.

Somatic mutations of the APC gene

Extensive screening for somatic mutations of the APC gene has been carried out in colorectal tumors. Our group has screened 160 colorectal adenomas and carcinomas, including 87 from FAP patients, and found 98 somatic mutations (Nishisho *et al.*, 1991; Miki *et al.*, 1992; Miyoshi *et al.*, 1992; Ichii *et al.*, 1992, 1993). Thirty-nine of the 98 somatic mutations have been point mutations; 32 nonsense, seven missense. Twenty-one of 39 occurred at C residues; nine were at CpG sites, in a ratio similar to that observed in germ-line mutations. Fifty-six were frameshift mutations consisting of 42 deletions and 14 insertions. Three were intronic sequence deletions. Like the germ-line mutations, most (91 of 98) of these somatic mutations are predicted to truncate the APC products.

We have also detected somatic mutations of the APC gene in 13 of 91 gastric cancers examined and in three of 10 pancreatic cancers (Horii *et al.*, 1992a, b; Nakatsuru *et al.*, 1992). Unlike germ-line mutations in FAP patients or somatic mutations in colorectal tumors, a half of the mutations detected in gastric cancers were missense mutations. Furthermore, three separate somatic mutations were detected in each of two gastric carcinomas; this finding is interesting because among the nearly 100 colorectal tumors examined, not one has been found to carry three mutations. Missense mutations in gastric carcinomas may indicate that one or more of specific mutagens, possibly in food participate in genetic alterations of gastric mucosal cells.

Early genetic events during of colorectal carcinogenesis

To examine early genetic events during colorectal carcinogenesis, we searched for genetic alterations in 75 adenomas from seven patients with familial polyposis coli (FAP) and in 64 sporadic colorectal tumors (63 carcinomas and one adenoma). We investigated germ-line and somatic mutations in the APC gene, somatic mutations in the K-ras (codons 12, 13, and 61) and p53 genes (exons 5–8), and loss of heterozygosity on the short arm of chromosome 8. Thirty-two FAP adenomas carried detectable somatic mutations in the APC gene. When the adenomas developed in FAP patients were divided into three groups based on their size (<3 mm, 3–10 mm, or >10 mm) or their pathohistological grade (mild, moderate, or severe dysplasia), the somatic APC mutations were detected even in adenomas of <3 mm with moderate dysplasia while K-ras mutations were rare in these tumors. Hence, inactivation of both alleles of the APC gene is considered to play a significant role in development of colorectal adenoma. Mutation of the p53 gene was observed in only two adenomas and loss of heterozygosity (LOH) on

8p22 was detected in none. These results imply that a second "hit" in the APC gene, but not necessarily mutation in K-ras or p53, is an important and critical event for formation of a colorectal adenoma (Ichii *et al.*, 1993).

Genetic diagnosis of lymph-node metastasis of patients with colorectal cancer

To apply the information of genetic alterations in colorectal cancers to clinical field, we have applied the mutant allele-specific amplification (MASA) method for detection of micrometastases to lymph nodes which are histologically diagnosed negative. To examine prognostic significance of genetically-detectable tumor cells in regional lymph nodes of colorectal cancer patients, we screened 120 colorectal cancers without histologically-detectable lymph node metastasis, for K-ras (codon 12, 13, and 61) or p53 mutations (exons 5-8) and examined corresponding regional lymph node at the genetic level by the MASA method. All of the patients, who had no histologically-detectable lymph-node metastasis but had distant metastases or local recurrences after surgery, were genetically diagnosed lymph node positive. Genetically-positive lymph node metastases were found in all of the patients in the recurrence group, but in only 22.7% of those in the recurrence-free group, with a statistically significant difference ($p<0.0001$) (Hayashi *et al.*, 1995). Hence, Genetic diagnosis of lymph node metastasis may become a useful prognostic factor and be a selective marker for post-operative intensive adjuvant chemotherapy.

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