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Nuclear Oncogene Amplification or Rearrangement is not Involved in Human Colorectal Malignancies

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Abstract—*We have examined 44 cases of human colonic and rectal carcinomas for structural rearrangement and amplification of c-myc, N-myc, L-myc, c-myb and p53 oncogenes. DNA hybridization showed evidence of c-myc amplification in only one of the samples tested. In addition, the same tumour also showed a rearrangement immediately 3′ to the c-myc locus. No rearrangement could be found at the c-myc locus in the other 43 cases. Moreover, our molecular analysis of N-myc, L-myc, c-myb and p53 genes indicated no relevant alteration of the copy number and/or genomic structure of these nuclear oncogenes. Thus, at least in human colorectal malignancies, it is unlikely that nuclear oncogene structural alterations and/or amplification plays a major role in tumour induction or progression.*

INTRODUCTION

CELLULAR proto-oncogenes have been identified by their homology to the oncogenic sequences of acutely transforming retroviruses or by DNA-mediated gene transfer techniques [1, 2]. Evidence suggests that proto-oncogene-encoded proteins may represent components of the signalling pathways through which growth factors and mitogens exert their effect on cells. By such pathways, growth stimulating signals are conveyed through membrane receptors and cytoplasmic proteins to the nucleus, where a proliferative response may be induced. As part of this picture, nuclear proto-oncogene products are surmised to play a central role in regulating cell proliferation and differentiation [3, 4].

Although the relationship between cellular proto-oncogene activation and the development of the neoplastic state remains to be elucidated, it appears that structural or regulatory alterations of c-onc genes are involved in tumour formation and/or progression. Modifications in the pattern of nuclear oncogene expression by gene amplification, retroviral insertion or chromosome translocation have been frequently demonstrated in several human tumours and tumour cell lines [5–10].

The myc proto-oncogene family has three well-characterized members, c-myc, N-myc and L-myc, which share similar structural features [11–14]. Nevertheless, this gene grouping not only reflects a structural kinship but also suggests that these kindred sequences may have related functions. The c-myc oncogene has been found to be translocated in lymphomas [5, 15] and amplified in the HL60 promyelocytic leukaemia cell line [16]. In addition, this oncogene is amplified and overexpressed in mammary, lung and colon carcinoma cell lines [7, 9, 10]. Particularly interesting is the finding of the amplification of N-myc and L-myc genes, which have been correlated to the clinical stage and the progression of neuro- and retinoblastomas and lung cancers [10, 17, 18]. Increased expression of the c-myc gene seems to occur frequently in human colonic carcinomas [19–20]. Amplification of c-myc with overexpression of this gene has initially been found in a human colonic cancer cell line with neuroendocrine properties (Colo 320) but there was no evidence of rearrangement or amplification of the myc locus in a group of 29 primary human colon carcinomas [19].

We carried out similar analyses on c-myc and four other nuclear oncogenes (N-myc, L-myc, c-myb, p53) to evaluate the real importance of structural alterations of such genes in human colorectal malignancies.

MATERIALS AND METHODS

Normal and neoplastic tissues

Normal and neoplastic tissues were collected from 24 patients with adenocarcinoma of the colon and from 20 patients with adenocarcinoma of the rec-

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tum. None of the 44 patients had undergone treatment prior to surgery. Neoplastic tissues and normal mucosa (dissected near the margins of resection) were removed by the attending pathologist immediately after surgery. Specimens were frozen in liquid nitrogen as soon as possible after surgical excision and were subsequently stored at −80°C until analysed. In cases where the normal colonic mucosa was unavailable, 20 ml of peripheral blood was taken in the presence of heparin. Lymphocytes were then separated by sedimentation through Ficoll/hypaque and frozen in liquid nitrogen.

Southern blotting

High molecular weight DNA was isolated as described previously [22]. DNAs were digested with restriction endonucleases as recommended by the manufacturers (Amersham, Boehringer, Milano, Italy), electrophoresed in 0.7% agarose gels and transferred to Gene Screen Plus (New England Nuclear) by the Southern method [23]. Hybridization was carried out at 37°C for 18 h in a buffer containing 50% formamide and 2×10^6 cpm/ml of a ^{32}P labelled probe (1.8×10^9 cpm/μg). Blots probed with c-myc, N-myc, L-myc, c-myb, c-mos and p53 were washed twice for 5 min in 2 × SSC at room temperature, twice for 30 min in 2 × SSC–1% SDS at 65°C, and twice for 30 min in 0.1 × SSC at room temperature. The filters hybridized to v-Ki-ras were washed under moderately stringent conditions (twice for 30 min in 1 × SSC–0.1% SDS at 55°C and once for 30 min in 1 × SSC at 55°C). The filters were then autoradiographed using intensifying screens at −70°C for 18–72 h.

Probes

The following restriction enzyme-generated DNA fragments were used as hybridization probes: the 1.6 kb Cla I-EcoRI fragment of pHSR-1 (c-myc) [7], the 1 kb EcoRI–BamHI fragment of pJ B327 (L-myc) [14] (a kind gift from Dr. J. Minna), the 1 kb EcoRI fragment of HiHi3 (v-Ki-ras) [24], the 2.7 kb EcoRI fragment of pHM2A (c-mos) [25], the 1.8 kb EcoRI fragment of p53–H13 (p53) [26] and a 1.2 kb PstI fragment of a c-DNA clone of c-myb [27], a kind gift from Dr. Pierotti. DNA probes were prepared from plasmid after resolution in low-melting-point agarose gel. DNA fragments were radiolabelled with ^{32}P dCTP by the random primer method of Feinberg and Vogelstein (Amersham) [28].

RESULTS

We studied the organization of five nuclear proto-oncogenes, c-myc, N-myc, L-myc, c-myb and P-53 in the DNA of 44 freshly obtained colonic and rectal carcinomas. Peripheral lymphocytes or normal colonic mucosa DNAs of the same patient were used as a reference to measure the extent of amplification. In addition, the Ki-ras and c-mos genes were used as an internal control for the amount of DNA transferred to the filters.

Southern blotting analysis of the c-myc locus structure was performed using EcoRI or HindIII restriction endonucleases, which cut the DNA outside the c-myc gene. Representative results are seen in Fig. 1. In all DNAs tested (with the exception of sample 181), the human c-myc probe detected 12.5 kb EcoRI and 11.8 kb HindIII fragments, which have been shown to be the germ-line human c-myc gene fragments. Thus, the c-myc oncogene does not appear to be rearranged in any of these tumours.

However, compared to the normal colonic mucosa, an extensively labelled band corresponding to the c-myc gene was revealed in DNA of sample 181 digested with EcoRI or HindIII. The other DNAs were labelled by the probe at intensities corresponding to single-copy sequences in control cells.

The degree of c-myc amplification in sample 181 was estimated from diluted DNA. As shown in Fig. 2a, a 10-fold dilution of sample 181 DNA was required to obtain an intensity corresponding to that of the control DNA, indicating a 10-fold amplification of the c-myc gene. However, the c-mos gene, located close to c-myc on chromosome 8 [25] was not amplified or rearranged in sample 181 DNA (Fig. 2b). The difference in the hybridization signals between c-myc and c-mos supported the interpretation that a specific amplification involving the c-myc gene occurred in this tumour. Owing to a lack of material it was impossible to perform any cytogenetic analysis of sample 181, with respect to double minute chromosomes and homogeneous staining regions. Furthermore, the third exon-c-myc probe detected a novel c-myc-related 23-kb-HindIII fragment in carcinoma 181.

In an attempt to better characterize the rearranged c-myc locus of this tumour and to exclude the presence of HindIII restriction site polymorphism, DNAs from 181 and from corresponding normal mucosa (sample 182) were digested with the enzymes SacI, XbaI, PstI and BglII, which cut at different distances from the 3′ end of the c-myc gene. Abnormal fragments of 5 and 6.2 kb in size were observed with BglII and PstI, respectively (Fig. 3).

Thus it was possible to map the beginning of the rearrangement between the conserved XbaI site and the abolished PstI site at the 3′ end of the c-myc locus.

With all the restriction enzymes employed the intensities of the normal c-myc fragments were amplified about 10-fold with respect to the abnormal ones.

To investigate whether the c-myc closely related oncogene N-myc was amplified or structurally

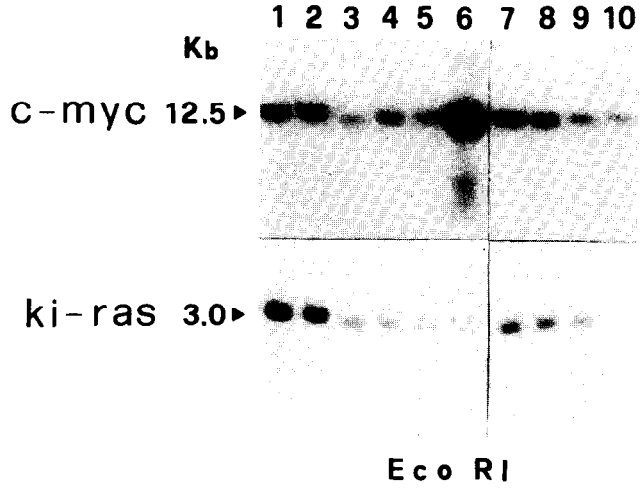

Fig. 1. Eco RI digests of DNAs from a representative group of colorectal carcinomas (lanes 2,4,6,8,10) and from corresponding normal tissues (lanes 1,3,5,7,9) hybridized with a c-myc specific probe. The same blot was rehybridized with a Ki-ras probe, indicating relative single-gene copy levels. Relative to the Ki-ras and normal colonic mucosa (sample 182, lane 5), one tumour DNA (sample 181, lane 6) showed an intense signal corresponding to the 12.5 kb-Eco RI c-myc fragment, indicating that it is amplified in this fragment. kb = *kilobases.*

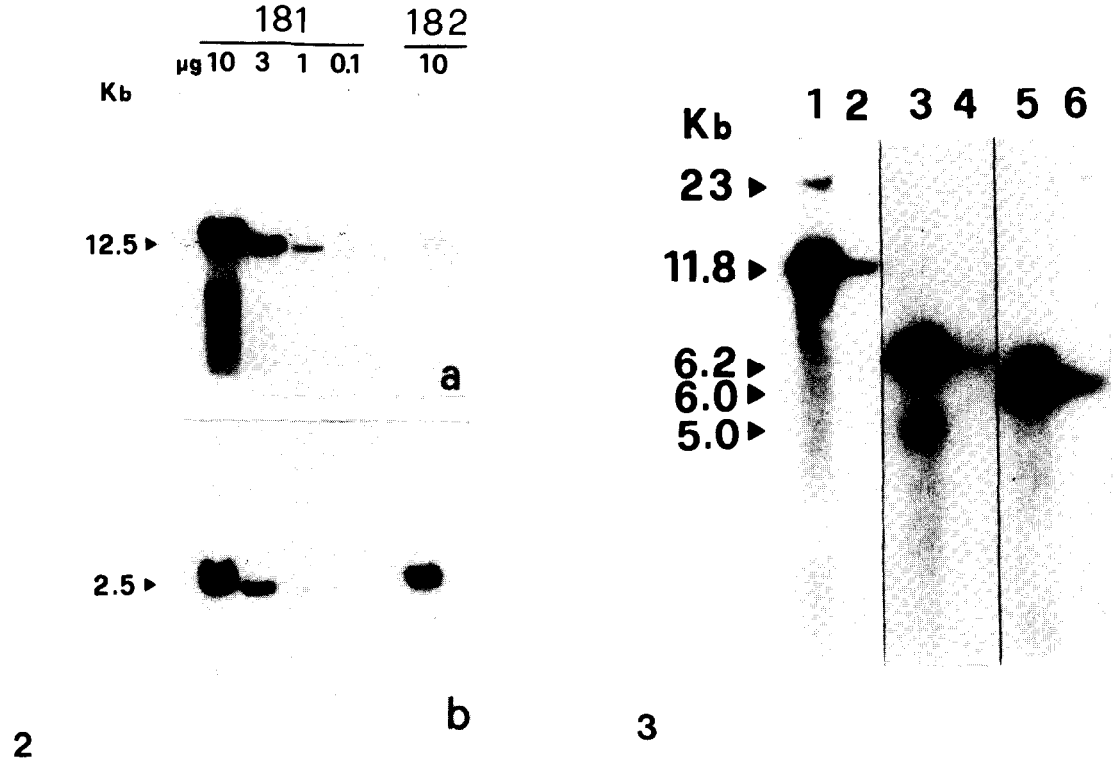

Fig.2. (a) Estimation of the degree of c-myc gene amplification in sample 181. Southern blotting of various amounts of DNA from tumour 181 was compared to 10 µg of DNA from the corresponding normal tissue. The results indicate that sample 181 has a greater than 10-fold increase in copy number, relative to sample 182. (b) Southern blot analysis of c-mos gene. Rehybridization of the same filter with a human specific c-mos probe showed no significant differences in the intensities of the 2.5 kb fragments obtained from equal amounts (10 µg) of 181 and 182 DNAs. kb= *kilobases.*

Fig. 3. Localization of the c-myc locus rearrangement in tumour 181. Ten micrograms of DNA from tumour 181 (lanes 1,3,5) and from the normal colonic mucosa of the same patient (sample 182, lanes 2,4,6) were digested with HindIII (lanes 1,2), BglII (lanes 3,4) and Pst I (lanes 5,6). Hybridization of blots with a third exon-c-myc probe detected novel myc related fragments only in sample 181 (23 kb in HindIII-digested DNAs, 5 kb in BglII-digested DNAs and 6.2 kb in PstI- digested DNAs). kb = *kilobases.*

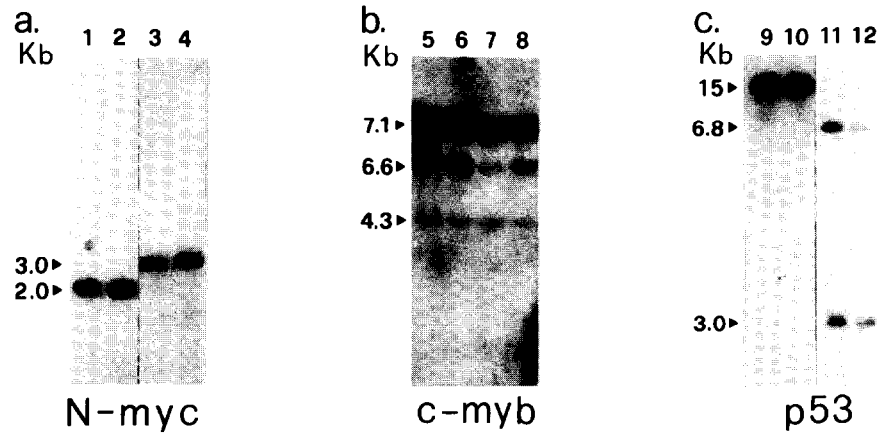

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Fig. 4. Southern blot analysis of the N-myc, c-myb and p53 loci in DNAs from colorectal carcinomas (lanes 1,3,5,7,9,11*) and corresponding normal controls (lanes* 2,4,6,8,10,12*). (a) Eco RI (lanes* 1,2*) and BglII (lanes* 3,4*) digested DNAs hybridized with a specific N-myc probe. (b) Hybridization analysis of four DNAs after Eco RI (lanes* 5,6,7,8*) digestion using a human c-myb probe. (c) Autoradiograms of Eco RI (lanes* 9,10*) and HindIII (lanes* 11,12*) digested DNAs hybridized with a p53 probe.* kb = *kilobases.*

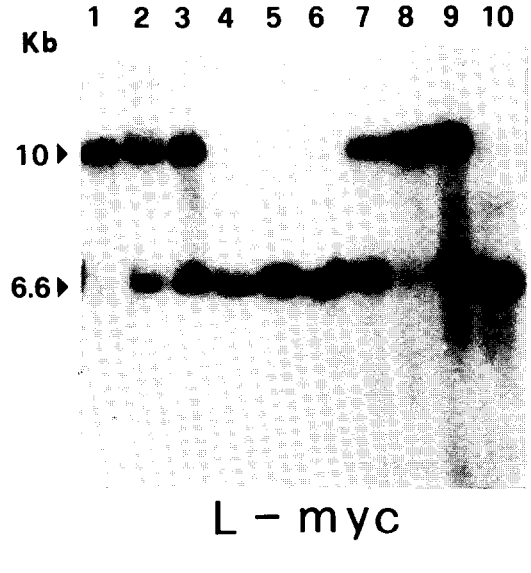

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Fig. 5. Southern blot analysis of representative genomic DNAs from colorectal adenocarcinomas digested with Eco RI and probed with the L-myc gene. The 10 kb *and* 6.6 kb *positions represent the Eco RI genomic L-myc fragments.* kb = *kilobases.*

modified in colorectal cancers, Southern blots of EcoRI and BglII digested DNA were hybridized with a homologous specific probe. A similar analysis was carried out hybridizing EcoRI digested DNAs with a human L-myc probe. Hybridization of blots with these two probes did not reveal any alteration in the relative intensity or sizes of the bands in tumour samples as compared to normal colonic mucosa or peripheral lymphocytes (Fig. 4a). As previously described [14], EcoRI endonuclease revealed a restriction fragment length polymorphism of L-myc gene defined by two alleles (10.0 and 6.6 kb), equally represented in the 44 patients examined (Fig. 5).

For Southern blot analysis of the c-myb locus, genomic cellular DNAs were digested with HindIII restriction endonuclease and hybridized with a radioactive molecular clone of the human c-myb gene. Our results revealed the presence of three normal c-myb-hybridizing bands of 4.3, 6.6 and 7.1 kb in all the samples tested (Fig. 4b) with no evidence of gene amplifications. Further analysis of DNA fragments generated by HindIII and EcoRI restriction enzymes hybridized to a ^{32}P labelled p53 probe revealed in all tumours examined only specific bands (3.0 and 6.8 kb for HindIII-digested samples, 15 kb for the EcoRI-digested samples) (Fig. 4c). Thus, there was no detectable gross alteration in the genomic organization or in gene copies of the p53 gene in tumour specimens as compared to normal ones. In addition, hybridization of blots to probes of human c-mos did not reveal in any case the variant EcoRI allele of 5 kb observed in patients with breast cancers [29] (data not shown). Thus, it appears that this rare allele at the c-mos locus may not significantly contribute to colorectal tumour susceptibility.

DISCUSSION

Although the normal cellular functions of proto-oncogenes are not yet well understood, it is a widely accepted hypothesis that activated c-onc genes play an important role in the malignant process [1, 2]. Recent evidence suggests that analysis of these genes and of their products may have important benefits for cancer patients in the development of more precise diagnostic tools and in providing a better prognosis. Thus, the two best known cases of oncogene activation by chromosomal translocation, the myc/Ig juxtaposition in Burkitt's lymphoma [30] and the transposition of c-abl from chromosome 9 to 22 in pH′ chromosome-positive chronic myelogenous leukaemia [31], are useful molecular markers in these malignancies. Moreover, recent findings show that oncogene amplification may be associated with tumour progression in specific human cancers. Thus, in neuroblastoma N-myc amplification was found at a high frequency and exclusively associated with stages III and IV [17], suggesting that such an alteration is involved in progression of tumours to a more malignant form. In addition, the three members of the myc family were found to be amplified in small-cell lung carcinomas [10, 14, 32] and c-myc amplification was reported to be related to an aggressive histological subtype [10]. More recently, Slamon *et al.* [33] showed that amplification of the HER-2/neu oncogene occurs relatively frequently in breast cancer, and that it is associated with disease relapse and overall patient survival.

The aim of our study was to determine the actual prevalence of gene amplifications and structural rearrangements of c-myc and other nuclear oncogenes in human colorectal malignancies. We chose to analyse the structure of these genes because of their supposed involvement in regulating the terminal phases of the mitogenic pathway [34]. In fact, in some experimental models, nuclear oncogenes seem to be implicated in the induction of the transformed phenotype [35]. Confirming previous findings [19, 21], our data indicate that c-myc amplification occurred very rarely, only in one of the 44 carcinomas examined. In addition to c-myc amplification, the same tumour also showed a genomic rearrangement immediately 3′ to the c-myc locus. However, the small proportion of rearranged c-myc copies observed in sample 181 is probably a consequence of the amplification process, and its functional importance remains to be elucidated. Therefore, previous reports [19, 21] and our findings indicate that the elevated hyperexpression of the c-myc mRNA, frequently observed in human colorectal malignancies, cannot be related to gene amplification or obvious structural changes at the c-myc locus. However, we cannot rule out that point mutations or small deletions or insertions, not detectable by the analytical methods we used, could affect the c-myc locus in these malignancies. Another possible explanation of c-myc mRNA hyperexpression is that other oncogenes activated in the development of colorectal tumours may alter the regulation of c-myc transcription or may increase c-myc mRNA stability. In our study we found no evidence of amplification or gross rearrangements of the c-Ki-ras and the c-mos proto-oncogenes (data not shown), although occasional examples of amplified c-Ha-ras and c-Ki-ras genes [35, 36] as well as overexpression of the p21 ras proteins in colon polyps and tumours have been reported [37–39]; however, as in other studies [40, 41] using the DNA transfection technique on NIH 3T3 cell line, we found a remarkable incidence of Ki-ras activation in these tumours: three out of 12 carcinomas tested (unpublished results).

N-myc is described as undergoing gene structure modifications, and/or mRNA hyperexpression in tumours of neuroendocrine origins. However, no

information is available about its involvement in colorectal cancers. Since gene amplifications are the almost unique genomic structural alteration described, we analysed this phenomenon in our colorectal carcinoma samples. Our data exclude the possibility that N-myc amplification occurs with a significant frequency in these tumours. Moreover, we found no evidence of major gene rearrangement, although more detailed analyses are needed to confirm this preliminary finding. Since current studies suggest that individual members of the myc family may have important roles within specific cell lineages, we carried out a molecular analysis also at the L-myc locus. Hybridization of Southern blots did not reveal any alteration in the relative intensity of the bands in tumour samples as compared to normal counterparts. In addition, the frequency of the two EcoRI–L-myc alleles in the 44 patients examined were as previously described [14].

A previous report [42] described amplification and hyperexpression of the c-myb gene in two cell lines independently derived from a single adenocarcinoma of the colon. Due to the high structural complexity of the c-myb locus [43], we were able to analyse only amplification of this gene and eventually major rearrangements. Again, our analysis did not reveal any structural modification affecting the c-myb gene in colorectal carcinomas. We do not know whether the amplified c-myb gene in the previously described colonic adenocarcinoma cell lines is a characteristic restricted to a small fraction of the colorectal tumours not represented in our samples. However, since oncogene amplification has mainly been found in cell lines of various origins, it is likely that in some cases such a structural abnormality could arise during the course of propagation of established cell lines.

We also studied the structural organization of the p53 gene, whose product has been detected in several human cancers [44]. It has been proposed that such a nuclear oncogene-encoded protein may play a role in cell cycle regulation, in the induction of immortality and possibly in the transformation of certain non-human cells [45–47]. Skin fibroblasts from patients with adenomatosis of the colon and rectum were shown to express elevated amounts of the p53 antigen [48] and the overexpression of this gene was supposed to be an early event, possibly associated with initiation and promotion of inherited colonic cancer. Our molecular analysis of the p53 gene indicated no relevant alteration in the copy number and/or size of this nuclear oncogene, suggesting the role of oncogene activation mechanisms other than amplification or rearrangement in colon and rectum tumours.

Thus, at least in human colorectal carcinomas, nuclear oncogene structural alterations and/or amplifications are unlikely to play a major role in tumour induction or progression. In addition, it appears that nuclear oncogene amplification or rearrangement cannot serve for diagnostic or prognostic purposes in this disease.

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