Moderate amplifications of the c-myc gene correlate with molecular and clinicopathological parameters in colorectal cancer

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Summary C-myc gene activation is a common event in multiple types of neoplasia and has been associated with different cellular processes relevant to the malignant transformation of cancer cells. C-myc gene amplification has been analysed in colorectal carcinomas by means of an innovative DNA fingerprinting method based on the arbitrarily primed PCR. This method requires a low amount of DNA, uses multiple internal controls and appears sensitive and reproducible. Clinicopathological and molecular correlates have been investigated in a series of 70 colorectal carcinomas. The incidence of c-myc amplification was 26%, ranging from two- to five-fold increase in copy number. C-myc amplification occurrence was more frequent in more advanced stages of tumour invasion (P < 0.001) and was associated with mutations in the p53 tumour-suppressor gene (P = 0.048). The presence of c-myc amplification was indicative of a shorter disease-free survival period but, because of its strong association with Dukes’ stage, its prognostic value is questionable.

Keywords: oncogene amplification; tumour progression; genetic alteration; arbitrarily primed PCR; DNA fingerprinting

After the initial finding of an amplified c-myc gene (also known as MYC) in different human cell lines (Collins and Groudine, 1982; Altalal, 1983; Little et al, 1983), oncogenic activation of the c-myc gene through amplification has been demonstrated in multiple types of cancer (reviewed by Bishop, 1991; Garte, 1993). Previous studies have shown that c-myc gene amplification and/or overexpression in colorectal cancer correlates with the degree of invasion (Kozma et al, 1994; Sato et al, 1994) and poor differentiation (Heerdt et al, 1991). Nevertheless, other authors have failed to find such associations (Erisman et al, 1988; Finley et al, 1989; Matsumara et al, 1990; Nagai et al, 1992; Smith et al, 1993). Although c-myc overexpression appears in an elevated proportion of tumours (66–90%) (Erisman et al, 1985; 1988; Matsumara et al, 1990; Smith et al, 1993; Sato et al, 1994; Wang et al, 1994), gene amplification, as determined by Southern (Erisman et al, 1985; Untawale and Blick, 1988; Finley et al, 1989; Matsumara et al, 1990; Heerdt et al, 1991) or dot-blot hybridization (Erisman et al, 1988; Nagai et al, 1992; Smith et al, 1993; Kozma et al, 1994; Wang et al, 1994) is less frequent, ranging from 0 to 30%. In addition to the intrinsic complexity of cellular processes, sample heterogeneity and methodological pitfalls are important factors that puzzle the comprehensibility of these divergences.

With the advent of the polymerase chain reaction (PCR) and in order to overcome the limitation of blot hybridization techniques (mainly, a high amount of genomic DNA is required), different quantitative PCR methods for c-myc analysis have been developed (Rheo-Mojuela et al, 1993; Watson et al, 1993; Sugimoto et al, 1994; Sestini et al, 1995). PCR co-amplification of the c-myc gene with an internal control did not render reliable results in our hands. Theoretical and practical considerations limit the accuracy of this type of technique, often generating irreproducible results (see review by Ferré et al, 1994).

The initial aim of this study was to determine the possible relationship between c-myc gene amplification and molecular and clinicopathological variables in a series of colorectal carcinomas. As only a limited amount of DNA was available from normal and tumour tissue (making the use of hybridization-type methods impossible) and in order to overcome the technical limitations described above, we have set up a PCR-derived method that uses multiple internal and arbitrary controls. This technique is based on the arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990), a fingerprinting-type technique using primers whose nucleotide sequence is arbitrarily chosen.

It has been demonstrated that AP-PCR is useful for the detection and isolation of tumour-specific allelic losses and gains (Peinado et al, 1992; Kohno et al, 1994; Achille et al, 1996), thus providing a molecular alternative to cancer cytogenetics. In addition, DNA fingerprinting by AP-PCR permitted the discovery of ubiquitous somatic genomic instability in a subset of colorectal tumours (Ionov et al, 1993). More recently, AP-PCR fingerprinting has been used to estimate the degree of genomic damage in colorectal tumours (Bocker et al, 1996; Arribas et al, 1997; Basik et al, 1997). We have observed that the rate of genomic damage in neoplastic cells, as determined by AP-PCR, may have important prognostic applications (Arribas et al, 1997). Two intrinsic features of AP-PCR are of special interest: (a) the amplified sequences proceed from randomly selected genome regions with no apparent bias for the chromosomal origin of the bands (Peinado et al, 1992; Yasuda et al, 1996); and (b) the amplification is quantitative in that the intensity of an amplified band is proportional to the concentration of its corresponding template sequence (Peinado et al, 1992; Perucho et al, 1995).
In our approach, primers are designed to amplify a fragment including the third exon of the human \( c-myc \) gene and PCR is performed under low-stringency conditions, as in AP-PCR. In consequence, the \( c-myc \) gene fragment is co-amplified with a number of arbitrary sequences that consistently appear in all the samples. These sequences are flanked by the same primers as the \( c-myc \) gene and competition among them generates a quantitative fingerprint (see scheme in Figure 1). Furthermore, we can expect

| Stage | Case | Age | Sex | DFS | Rec | Follow-up (months) | Dead | pS3 | ras | MMP | \( c-myc \) |
|-------|------|-----|-----|-----|-----|-------------------|------|-----|-----|-----|------|
| Dukes | 6    | 77  | M   | 62  | Yes | 94                | No   | +   | –   | –   | –    |
| A     | 44   | 77  | F   | 96  | No  | 96                | No   | +   | –   | –   | –    |
| A     | 51   | 67  | F   | 68  | Yes | 68                | No   | –   | +   | –   | –    |
| A     | 57   | 71  | M   | 83  | No  | 83                | No   | +   | –   | –   | –    |
| A     | 58   | 69  | M   | 78  | No  | 78                | No   | –   | +   | –   | –    |
| A     | 60   | 75  | F   | 43  | No  | 43                | No   | ND | –   | –   | –    |
| A     | 66   | 76  | F   | 44  | Yes | 80                | No   | +   | –   | –   | –    |
| A     | 83   | 73  | M   | 52  | No  | 52                | No   | –   | –   | –   | –    |
| A     | 89   | 39  | M   | 84  | No  | 84                | No   | –   | –   | +   | –    |
| A     | 119  | 61  | M   | 60  | No  | 60                | No   | +   | –   | –   | –    |
| A     | 135  | 79  | F   | 65  | No  | 65                | No   | –   | +   | –   | +    |
| A     | 180  | 55  | M   | 81  | No  | 81                | No   | +   | –   | –   | –    |
| B     | 33   | 61  | M   | 29  | Yes | 29                | Yes  | +   | –   | –   | –    |
| B     | 36   | 80  | F   | 27  | Yes | 27                | Yes  | +   | –   | –   | –    |
| B     | 42   | 77  | M   | 85  | No  | 85                | No   | +   | –   | –   | –    |
| B     | 45   | 76  | M   | 84  | Yes | 84                | No   | ND | –   | –   | –    |
| B     | 47   | 59  | M   | 87  | Yes | 87                | No   | +   | –   | –   | –    |
| B     | 48   | 81  | M   | 31  | Yes | 31                | Yes  | –   | –   | +   | –    |
| B     | 49   | 60  | M   | 62  | Yes | 62                | Yes  | –   | –   | –   | –    |
| B     | 55   | 68  | M   | 84  | Yes | 84                | No   | –   | +   | –   | –    |
| B     | 59   | 56  | M   | 70  | No  | 70                | No   | –   | +   | –   | –    |
| B     | 62   | 62  | M   | 79  | No  | 79                | No   | +   | –   | –   | –    |
| B     | 68   | 73  | M   | 34  | Yes | 34                | No   | –   | +   | –   | –    |
| B     | 78   | 62  | M   | 71  | Yes | 71                | No   | –   | +   | –   | –    |
| B     | 91   | 53  | F   | 28  | Yes | 28                | No   | ND | –   | +   | –    |
| B     | 100  | 60  | F   | 61  | No  | 61                | No   | ND | +   | –   | –    |
| B     | 118  | 70  | M   | 61  | No  | 61                | No   | +   | –   | –   | –    |
| B     | 121  | 66  | F   | 45  | Yes | 45                | Yes  | +   | –   | –   | +    |
| B     | 132  | 71  | M   | 59  | No  | 59                | No   | –   | –   | +   | –    |
| B     | 136  | 58  | M   | 19  | Yes | 19                | Yes  | +   | –   | –   | –    |
| B     | 150  | 69  | M   | 15  | Yes | 15                | Yes  | +   | –   | –   | –    |
| B     | 153  | 63  | M   | 49  | No  | 49                | No   | +   | –   | –   | –    |
| B     | 186  | 56  | M   | 44  | Yes | 46                | Yes  | –   | +   | –   | –    |
| B     | 190  | 63  | M   | 68  | No  | 68                | No   | –   | –   | –   | –    |
| B     | 193  | 68  | F   | 48  | No  | 48                | No   | +   | –   | –   | –    |
| C     | 27   | 63  | M   | 5   | Yes | 5                 | Yes  | +   | –   | –   | –    |
| C     | 56   | 76  | M   | 84  | No  | 84                | No   | +   | –   | –   | –    |
| C     | 94   | 56  | M   | 67  | Yes | 67                | No   | –   | +   | –   | +    |
| C     | 96   | 66  | M   | 20  | Yes | 20                | Yes  | –   | +   | –   | –    |
| C     | 101  | 61  | M   | 61  | No  | 61                | No   | +   | –   | –   | –    |
| C     | 117  | 93  | M   | 9   | Yes | 9                 | Yes  | +   | –   | –   | +    |
| C     | 134  | 62  | F   | 66  | No  | 66                | No   | +   | –   | –   | +    |
| C     | 137  | 71  | M   | 29  | Yes | 29                | Yes  | +   | –   | –   | +    |
| C     | 156  | 60  | M   | 30  | Yes | 57                | Yes  | ND   | –   | –   | –    |
| C     | 189  | 71  | F   | 6   | Yes | 6                 | Yes  | +   | –   | –   | –    |
| C     | 191  | 78  | M   | 3   | Yes | 3                 | Yes  | –   | –   | –   | +    |
| C     | 192  | 64  | M   | 78  | Yes | 78                | No   | +   | –   | –   | +    |
| C     | 201  | 54  | F   | 76  | No  | 76                | No   | –   | +   | –   | –    |
| C     | 202  | 53  | M   | 69  | No  | 69                | No   | +   | –   | –   | +    |
| C     | 213  | 48  | M   | 22  | No  | 22                | No   | –   | –   | –   | –    |
| D     | 65   | 64  | M   | 52  | Yes | 52                | Yes  | –   | +   | –   | +    |
| D     | 80   | 57  | M   | 9   | Yes | 9                 | Yes  | ND  | –   | –   | +    |
| D     | 125  | 56  | F   | 12  | Yes | 12                | Yes  | +   | –   | –   | +    |
| D     | 133  | 47  | M   | 21  | Yes | 21                | Yes  | +   | –   | –   | –    |
| D     | 182  | 69  | F   | 36  | Yes | 36                | Yes  | +   | –   | –   | –    |
| D     | 197  | 57  | M   | 5   | Yes | 5                 | No   | –   | +   | –   | +    |
| D     | 268  | 77  | M   | 5   | Yes | 5                 | No   | +   | –   | –   | –    |

*Only cases with available follow-up data are included. Sex: M, male; F, female; DFS, disease-free survival in months; Rec, recurrent disease; Dead: Yes, died of disease; No, alive with or without disease, dead of unrelated causes; pS3 and ras: +, with mutation; –, no mutation; ND, not determined; MMP, microsatellite mutator phenotype; +, instability in at least two out of five microsatellites sequences; –, no instability; c-myc: +, gene amplification; –, no gene amplification.*
that these sequences are of different chromosomal origin and, in consequence, they are useful as internal controls. As the rationale of this technique is based on the properties of AP-PCR, we have called this method targeted AP-PCR (TAP-PCR).

We have applied TAP-PCR to the allelic dosage of the c-myc gene in a series of colorectal tumours of which a limited amount of DNA was available. We have observed moderate c-myc gene amplifications in about one-quarter of the tumours. Amplifications of the c-myc gene occurred more frequently in advanced stages of tumour invasion and were associated with mutations in the p53 gene.

MATERIAL AND METHODS

Samples

Seventy colorectal carcinomas and paired normal tissue samples were obtained from the Human Tissue Cooperative Network (University of Alabama, Birmingham, USA). Phenotypic and genetic characteristics of these cases have been described elsewhere as part of a larger series (Capella et al, 1991; Peinado et al, 1993). The samples used in this study were selected from a collection of 181 cases based on availability of genomic DNA from tumour and paired normal mucosa. They were representative of the whole collection for all the molecular and clinicopathological parameters considered. Cases were pathologically staged using Astler–Coller modification of Dukes' classification system. Detailed histological study of a short series of cases revealed that in most cases (16 out of 17) more than 75% of the analysed tissue was composed of neoplastic cells. In a single case, stromal and normal epithelial cells constituted approximately 50% of the tissue. SW480 and DLD-1 cell lines (ATCC, Rockville, MD, USA) were used as positive and negative controls, respectively, for c-myc amplification. Cases with perioperative deaths and with insufficient follow-up were excluded from the survival analysis.

Molecular analyses

Genomic DNA was extracted by the phenol–chloroform method as described previously (Nakano et al, 1984). DNA was diluted to a concentration of 20 ng μl⁻¹ and 1 μl of each DNA was run in a 0.75% agarose gel and stained with ethidium bromide to verify its quality and concentration. When necessary, the DNA concentration was adjusted according to the ethidium bromide signal.

Mutations at codons 12 and 13 of the K-ras gene and 12 of the N-ras gene were detected and characterized by the artificial RFLP/PCR approach (Capella et al, 1991). p53 mutations in exons 4–9 were analysed using single-strand conformation polymorphism (SSCP) of PCR amplified products and characterized by direct cycle sequencing of the PCR product (Peinado et al, 1993). Genomic instability at simple repeated sequences was analysed in microsatellite sequences AP2, AP3, Mfd27, Mfd41 and Mfd47 as described previously (Ionov et al, 1993; Shibata et al, 1994). Cases showing instability in two or more microsatellites were considered to belong to the microsatellite mutator phenotype (MMP) pathway. In agreement with other reports (Thibodeau et al, 1993), tumours with low genomic instability (mutations in only one out of five microsatellite sequences analysed) present molecular and biological characteristics different from those with high instability but similar to the rest. In consequence, they were considered MMP negative.

TAP-PCR of c-myc

TAP-PCR reactions were performed in duplicate with 50 ng of genomic DNA, 125 μM each dNTP, 1 μM each primer (sense: 5’-AAAGAGGCAGGCTCCTGGCA-3’, antisense: 5’-TTCGTCGTTCGCAACAA-3’), 1 μCi [α-33P]dATP (Amersham, Buckinghamshire, UK) and 1.25 units of Taq polymerase (Boehringer Mannheim, Mannheim, Germany) in PCR buffer (10 mM Tris-HCl pH 8.0, 50 mM potassium chloride, 1.5 mM magnesium chloride) in a final volume of 25 μl. The reaction consisted of five low-stringency cycles (30 s at 94°C, 30 s at 50°C, 30 s at 72°C) and 35 high-stringency cycles (30 s at 94°C, 30 s at 65°C, 30 s at 72°C) and was carried out in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA). The product was diluted with formamide dye buffer, denatured for 3 min at 95°C and 3 μl were run on a 6% acrylamide 8 M urea sequencing gel at 55 W for 3 h. The gels were dried under vacuum at 85°C and exposed to radiograph film at room temperature without intensifier screen for 1–3 days. The amplified c-myc region corresponds to a fragment 470-bp-long, which includes

![Figure 1](image-url) Principles of TAP-PCR method. Conventional PCR amplification using specific primers (A) will produce a unique product corresponding to the sequence flanked by the primers and resolved as a unique band by gel electrophoresis (lane A). When low stringency conditions are applied, primers are likely to anneal to multiple regions of the genome (B and C) and many sequences are co-amplified in a competitive and reproducible fashion, including the one for which the primers are designed (lanes B and C). In this case, differences in copy number of the targeted sequence will be manifested as proportional changes in the amount of its corresponding TAP-PCR product band as resolved by electrophoresis. Right panel corresponds to a 6% polyacrylamide (non-denaturing) gel electrophoresis of c-myc amplification in the conditions described above. Lanes B and C contain the TAP-PCR products from a normal and its paired tumour DNA respectively. Gel was stained with ethidium bromide and colour inverted.
Figure 2  c-myc allelic dosage of SW480 and DLD-1 cell line DNAs. (Top) Duplicated TAP-PCR analysis of pure and mixed DNAs (mixture proportions are indicated at top) from these two cell lines. Solid arrow indicates the band identified as c-myc. Differences of intensity in this band are observed according to the mixture proportions as shown in the densitometric analysis at right. For calculation purposes, the intensity of five arbitrary bands (indicated by empty arrows) was monitored and used as reference. Densitogram is displayed for lanes marked with an asterisk. (Bottom) Southern blot hybridization analysis of the same cell line DNAs with a c-myc probe. In addition to the 7.5-kb band, a fainter band of approximately 5 kb (not shown) was identified in the SW480 DNA, probably corresponding to a genomic reorganization. The ethidium bromide staining of the digested genomic DNA before transfer is shown at left. TAP-PCR and Southern blot analyses were performed as described in Material and methods.

the third exon. The identity of the c-myc band was checked by simultaneous electrophoresis of the TAP-PCR product with the product of a conventional high-stringency PCR (annealing temperature, 65°C). The co-migrating band was isolated, cloned and sequenced using standard procedures (Peinado et al, 1992; Perucchini et al, 1995).

Differences in the intensity of bands between the tumour and its paired normal tissue were ascertained by direct eye inspection of the film. Relative increases in intensity of the c-myc band in the tumour sample compared with its normal sample was considered a symptom of gene amplification compared with the co-amplified arbitrary bands. In addition, in order to have an objective measurement of the magnitude of the change, films were scanned and the intensity of bands quantified using Phoretix 1D software (Newcastle upon Tyne, UK). The intensity of each c-myc band was divided by the sum of five arbitrary bands (Figure 2) in order to have a relative measure when the overall intensity of the lanes was not comparable. The ratio between normal tissue and tumour tissue was used to determine the degree of amplification of the c-myc gene in each case.

Method assessment

To test the sensitivity of this technique, duplicated serial dilutions of the SW480 DNA in the DLD-1 DNA were analysed using TAP-PCR and Southern blot hybridization. TAP-PCR was performed as described earlier. For Southern blot analysis, 10 μg of DNA was digested with Xba1 restriction endonuclease (New England Biolabs, Beverly, MA, USA) and electrophoresed on a 0.7% agarose gel. DNA denaturation and transfer to a blotting membrane (Quiagen, Santa Clarita, CA, USA) was performed using standard procedures (Sambrook et al, 1989). The filter was hybridized overnight with a c-myc probe labelled using the random primer method (Prime-It, Stratagene, La Jolla, CA, USA) with [α-32P]dCTP (Amersham). The probe was obtained by cloning a PCR product generated with the same primers used for TAP-PCR. The probe was sequenced to confirm its identity.

Statistical analysis

Statistical analysis was performed using the chi-square test, ANOVA or unpaired t-test as appropriate. Contingency tables were analysed using Fisher’s exact test or the chi-square test. Fifty-seven cases were included in the follow-up study (Table 1). Given the limited size of our series with complete follow-up and taking into account that five cases presented a late recurrence of the disease (6 or 7 years after surgery), to determine the potential application of c-myc amplification as a prognostic factor it was considered more appropriate to use the disease-free survival interval rather than the overall survival. Disease-free survival distributions were calculated using the Kaplan–Meier method and
analysed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. Statistical analysis was performed with SPSS software. All P-values are estimated from two-sided statistical tests.

RESULTS

Method assessment

Sequencing of the TAP-PCR band co-migrating with the specific PCR c-myc product (Figure 1) demonstrated its identity. Technique sensitivity was determined by serial dilutions of the SW480 DNA in the DLD-1 DNA and compared with Southern blot hybridization. Parallel results were obtained with both techniques (Figure 2). The maximum dilution of SW480 in DLD-1 that appeared to be distinguishable from uncontaminated DLD-1 was 1:31. As determined by densitometric analysis of the Southern blot hybridization, the c-myc gene is about 13.5 ± 0.8 (mean ± s.d.)-fold amplified in SW480 vs DLD-1, whereas using TAP-PCR it was 10.8 ± 0.7. This implies that a 1:31 dilution of SW480 DNA in DLD-1 DNA will correspond to a 1.4 c-myc amplification for DLD-1. As shown in Figure 2, TAP-PCR analysis is able to resolve such tiny differences, indicating that the sensitivity of the method is below a 1.5-fold amplification.

c-myc amplification in colorectal carcinomas

Taking into account the sensitivity of the technique and that our tumour samples were likely to contain a significant proportion of non-tumour cells, a minimal 1.5-fold increase in intensity in the tumour compared with the normal was required to be considered as an amplification. An intensity of the c-myc band in the tumour tissue vs its paired normal tissue (> 1.5-fold) was observed in 18 out of 70 cases analysed (26%). An illustrative example is shown in Figure 3. The amplification ratio ranged from two- to fivefold with an average of 3.05 ± 1.02 (mean ± s.d.). Owing to the heterogeneous proportion of tumour cells in a given sample, tumours have been classified as positive or negative for c-myc amplification, taking into account that we have a minimal estimation of the amplification ratio. Clinopathological and molecular associations of the c-myc amplification are summarized in Table 2. Lymph node-positive cases showed a higher incidence of c-myc amplification (47%) vs no lymph invasion (10%) (P = 0.0005). No differences were observed between Dukes’ stages C and D. In tumours containing a mutation in the p53 gene, c-myc was more often amplified (P = 0.0484). No significant correlation was found with other variables such as age, sex, race, tumour differentiation and location and presence of mutations in the ras gene. None of the eight tumours displaying microsatellite instability contained an amplified c-myc; nevertheless, the differences did not reach statistical significance.

Univariate survival analysis for the different parameters studied (Table 3) indicated that advanced Dukes’ stages and poor differentiation were markers of bad prognosis. Although cases with amplification of the c-myc gene also had a poorer disease-free survival (log-rank, P = 0.0628), due to its strong association with more advanced stages, it does not constitute an independent prognostic factor as determined by multivariate Cox analysis (Table 3).

Table 2  C-myc amplification in colorectal carcinomas

| Parameter | c-myc amplification* | Significance |
|-----------|----------------------|--------------|
| Race      |                      |              |
| Black     | 3/16 (19)            | 0.5050       |
| White     | 13/48 (19)           |              |
| Sex       |                      |              |
| Female    | 6/20 (30)            | 0.6038       |
| Male      | 12/50 (24)           |              |
| Dukes’ stage |                  |              |
| A–B       | 4/40 (10)           | 0.0024       |
| C         | 9/19 (47)            |              |
| D         | 5/11 (45)            |              |
| Lymph node invasion |          |              |
| Negative  | 4/40 (10)           | 0.0005       |
| Positive  | 14/30 (47)           |              |
| Location  |                      |              |
| Left      | 12/38 (31)           | 0.1220       |
| Right     | 4/27 (15)            |              |
| Differentiation |            |              |
| Well–Moderate |          |              |
| Poor      | 12/51 (23)           | 0.6639       |
| 3/10 (30) |                      |
| ras mutation |                  |              |
| Negative  | 14/46 (30)           | 0.2109       |
| Positive  | 4/24 (17)            |              |
| p53 mutation |                  |              |
| Negative  | 3/24 (12)            | 0.0484       |
| Positive  | 14/40 (35)           |              |
| MMP       |                      |              |
| Negative  | 18/62 (29)           | 0.0770       |
| Positive  | 0/8 (0)              |              |

*Numbers indicate cases displaying c-myc amplification respect the total for each category. Numbers in parentheses indicate percentages. *Microsatellite mutator phenotype.
be concluded that TAP-PCR is a reliable and sensitive method for the allelic dosage of c-myc. As the pattern is very reproducible from individual to individual, concomitant analysis of the paired normal mucosa is not absolutely necessary.

Nevertheless, in order to discard a polymorphic differential PCR amplification of one or many of the bands, it is highly recommended to use normal tissue from the same patient as the control. Owing to the intrinsic instability of the cancer cell genome, use of multiple and diverse reference loci is an indispensable condition. In consequence, one of the properties of AP-PCR, the random chromosomal origin of the co-amplified bands (Peinado et al, 1992; Yasuda et al, 1996), is of special relevance to guarantee the accuracy of the quantification.

The incidence of c-myc amplification in our series of colorectal tumours is in the upper range (about 25%) of the data reported in the literature, in agreement with Heerdt et al (1991) and Wang et al (1994). As the amplifications we have seen are moderate (three-fold in average), a lack of sufficient sensitivity may explain the failure of other studies to detect such gains. Although not significant, we have observed a lower incidence of c-myc amplification in the right-side colon (Table 2). This is in agreement with a previous study in which c-myc overexpression was more frequent in the left colon (Rothberg et al, 1985). In fact, these authors have already defined two pathways for sporadic colorectal tumours in relationship to the familial syndromes. Tumours behaving like hereditary non-polyposis colorectal cancer (HNPPC) were located at the right side and did not overexpress c-myc, whereas tumours behaving like familial polyposis coli were left sided and showed c-myc activation. In agreement with this observation, more recent evidence has shown that a significant proportion of the right-side-located tumours progress through a different molecular pathway characterized by a DNA mismatch repair-deficient machinery (Aaltonen et al, 1993; Ionov et al, 1993; Lothe et al, 1993; Peltomaki et al, 1993; Thibodeau et al, 1993; Peruch et al, 1994). These tumours do not show c-myc amplification (Table 2) and, in consequence, might be responsible for this trend.

Although there is a general agreement that c-myc amplification is more frequent in more aggressive tumours, including colorectal, gastric and lung (Heerdt et al, 1991; Yokota et al, 1986; Little et al, 1983; Shibuya et al, 1985), because of sample and technical heterogeneity data are rarely comparable. Tumours depicting c-myc amplification show a poorer disease-free and overall survival. Unfortunately, the relevance of this association has a doubtful application. The clear correlation with advanced Dukes’ stages indicates the dependency between both variables. This observation also holds for overall survival. In consequence, assessment of c-myc dosage status does not seem to be useful as a prognostic factor. c-myc amplification is one of the multiple alterations that accumulates during the tumour progression and its late appearance suggests that it does not play a significant role in the transformation of the cell. Nevertheless, experimental evidence shows that c-myc activation (which is not necessarily produced by gene amplification) has a direct and important implication in multistage carcinogenesis (Field and Spandidos, 1990). The apparent irrelevance, at clinical level, of c-myc activation in colorectal cancer is in a way expected and explained, at least in part, by its late occurrence. It can be hypothesized that either the activation mechanism (amplification) is a late event, or the selective advantage that it confers to the tumour cell is only displayed in late stages. This is based on studies that indicate that c-myc activation induces apoptosis (Evan et al, 1992). In consequence, for tumour cells,

### Table 3 Disease-free survival: univariate and multivariate analysis for clinical pathological and genetic parameters

| Prognostic factor                        | Univariate analysis | Multivariate analysis |
|-------------------------------------------|---------------------|-----------------------|
|                                           | Number of observations | 5-year survival (percentage) | Log-rank | P  |       |       |
| Dukes' stage (Astler-Coller)              |                     |                       |          |    |       |       |
| A–B                                       | 35                  | 71.1                  | <0.0001  |    |       |       |
| C                                         | 15                  | 51.8                  |          |    |       |       |
| D                                         | 7                   | 0                     |          |    |       |       |
| Degree of differentiation                 |                     |                       |          |    |       |       |
| Well–moderate                             | 43                  | 59.2                  | 0.0344   |    |       |       |
| Poor                                      | 7                   | 28.6                  |          |    |       |       |
| Location                                  |                     |                       |          |    |       |       |
| Left                                      | 29                  | 64.1                  | 0.5137   |    |       |       |
| Right                                     | 24                  | 58.3                  |          |    |       |       |
| ras mutation                              |                     |                       |          |    |       |       |
| Negative                                  | 38                  | 62.4                  | 0.7471   |    |       |       |
| Positive                                  | 19                  | 47.4                  |          |    |       |       |
| p53 mutation                              |                     |                       |          |    |       |       |
| Negative                                  | 19                  | 61.7                  | 0.7994   |    |       |       |
| Positive                                  | 32                  | 56.2                  |          |    |       |       |
| MMP                                       |                     |                       |          |    |       |       |
| Negative                                  | 50                  | 57.0                  | 0.9765   |    |       |       |
| Positive                                  | 7                   | 57.1                  |          |    |       |       |
| c-myc amplification                       |                     |                       |          |    |       |       |
| Negative                                  | 42                  | 61.1                  | 0.0628   |    |       |       |
| Positive                                  | 15                  | 45.7                  |          |    |       |       |

Overall survival analysis produced similar results (univariate log-rank analysis, $P = 0.0673$; multivariate Cox analysis, hazard ratio = 1.2, $P = 0.7459$).

### DISCUSSION

Here, we report a variation of the AP-PCR fingerprinting technique by introducing a bias in the design of the primers towards the amplification of a particular sequence. This variation makes this technique suitable for the allelic dosage of specific genomic sequences. A similar approach has been used to isolate cDNA members of a gene family (Stone et al, 1994) and to quantify collagenase expression in tumour cells (Vinayals et al, submitted). The quality of the technique has been assessed by performing reproducibility and sensitivity tests. Finally, it has been validated by comparison with Southern blot hybridization analyses. The c-myc copy number in the SW480 cell line was of the same order when measured using Southern and TAP-PCR and in the same range as previously described (Untawale and Blick, 1988). It can
apoptosis overriding by activation/inactivation of other factors (see Stewart, 1994 for review) would be a prerequisite before c-myc amplification can occur. In fact, this hypothesis is supported by our observation that c-myc amplification is associated with mutations in the p53 gene (one of the inducers of apoptosis). In this case, p53 inactivation [that presumably occurs in the adenoma–carcinoma transition (Fearon and Vogelstein, 1990)] will play a double role to facilitate c-myc activation, on one side by inducing apoptosis abrogation (Yonish-Rouach et al., 1991; Shaw et al., 1992) and on the other by overriding of the genomic stability controls (Lane, 1992) that might prevent gene amplification (Livingstone et al., 1992; Yin et al., 1992). The positive correlation between c-myc activation and mutations in the p53 gene (Table 2) supports this hypothesis.

Although we have stated gene ‘amplification’ as all copy number increases observed at the c-myc level, this should be regarded with caution, especially in view of the modest nature of such increases. In addition, random screening of the cancer cell genome by AP-PCR has revealed that many anonymous bands located in chromosome 8 (as it is the c-myc gene) also display frequent gains, which in some cases are associated with c-myc amplification (two out of five) (unpublished data). This implies that the ‘amplified’ fragment may include a wide chromosomal region. If this is the case, such gains might affect a large region, if not all, of chromosome 8. This argument has relevant implications for two reasons: first, the number of genes displaying gains would be very high, and, in addition to c-myc, many of these genes may affect tumor behaviour. Second, the mechanism causing such imbalances is probably different from the one producing small regional amplifications. Precise characterization of the involved chromosomal fragment should answer such questions. Interestingly, extensive AP-PCR genome analysis of human cell lines carrying amplified c-myc genes showed that all of them displayed concurrent amplification of other DNA fragments mapped to chromosome 8 (Okazaki et al., 1996). Okazaki et al. (1996) hypothesize that these sequences are part of an amplification unit that includes the c-myc gene. The total size of the amplified region in a small-cell lung carcinoma cell line was estimated to be 7.5 Mb.

A possible inaccuracy in our results is the quantification of the c-myc copy number in tumour samples. Heterogeneous population and contamination by normal cells might mask the real degree of amplification. For this reason, we have only classified tumours as positive or negative for c-myc amplification, taking into account that we have a minimal estimation of the amplification ratio. Although the technique we have used is sensitive enough to detect a relatively small amplification in a relatively small proportion of the cells, it is obvious that this limitation will affect in a lower grade more advanced tumours and consequently may mislead in the interpretation of the results.

In summary, TAP-PCR appears to be an appropriate technique to determine gene amplification. The low requirement of DNA template and the simplicity of the technique indicates that it may be especially useful in the analysis of small pieces that provide insufficient amounts of material for analysis using other techniques. Our study clearly demonstrates a significant incidence of c-myc amplification in colorectal cancer and its association with invasiveness. Owing to the complexity of c-myc function in cellular processes, further studies are required to elucidate the participation of c-myc amplification in aetiology and the consequences that it may have for tumour progression.

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