Characterization of newly established colorectal cancer cell lines: correlation between cytogenetic abnormalities and allelic deletions associated with multistep tumorigenesis

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Abstract

We have established a series of 20 colorectal cancer cell lines and performed cytogenetic and RFLP analyses to show that the recurrent genetic abnormalities of chromosomes 1, 5, 17 and 18 associated with multistep tumorigenesis in colorectal cancer, and frequently detected as recurrent abnormalities in primary tumours, are also retained in long-term established cell lines. Earlier studies by us and other investigators showed that allelic losses of chromosomes 1 and 17 in primary colorectal cancers predicted poorer survival for the patients ($P = 0.03$). We utilized the cell lines to identify specific chromosomal sites or gene(s) on chromosomes 1 and 17 which confer more aggressive phenotype. Cytogenetic deletions of chromosome 1p were detected in 14 out of the 20 (70%) cell lines, whereas allelic deletions for 1p using polymorphic markers were detected in 13 out of 18 (72%) informative cell lines for at least one polymorphic marker. We have performed Northern blotting, immunohistochemical staining (p53 mRNA, protein) and RFLP analysis using several probes including p53 and nm23. RFLP analysis using a total of seven polymorphic markers located on 17p and 17q arms showed allelic losses around the *p53* locus in 16 out of the 20 cell lines (80%), four of which were losses of the *p53* locus itself. In addition, seven cell lines (out of nine informative cases) also showed losses of the *nm23* gene, four with concurrent losses of the *p53* locus, while the remaining three were homozygous. In addition, five out of seven cell lines with *nm23* deletions were derived from hepatic metastatic tumours, and one cell line was obtained from recurrent tumour. A comparison between allelic deletions of 1p and functional loss of *nm23* gene revealed a close association between these two events in cell lines derived from hepatic metastasis. Following immunohistochemical staining, nine out of the twenty cell lines showed high levels (25–80%) of mutant p53, four showed intermediate levels (< 20%), and seven had undetectable levels of the protein. Of these seven, four showed complete absence of mRNA. Of the remaining three cell lines one showed aberrant mRNA due to germline rearrangement of the *p53* gene, whereas in two cell lines normal levels of mRNA were present. Nineteen of the 20 cell lines had normal germline configurations for the *p53* gene, while one showed a rearrangement. These data suggest that functional loss of *p53* and *nm23* genes accomplished by a variety of mechanisms may be associated with poor prognosis and survival. In addition, concurrent deletions of chromosome regions 17p, 17q and 1p were closely associated with high-stage hepatic metastatic disease. These cell lines with well-characterized genetic alterations and known clinical history provide an invaluable source of material for various biological and clinical studies relating to multistep colorectal tumorigenesis.

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Introduction

Colorectal cancer provides an excellent system to study genetic events associated with multistep tumorigenesis and in fact it is one of the most extensively studied epithelial tumour types at both cytogenetic and molecular levels (Vogelstein *et al*. 1988, 1989; Gerdes *et al*., 1989; Fearon and Vogelstein 1990; Jhanwar and Gerdes 1994; Gerdes *et al*. 1995; Jhanwar 1996). Most such studies, however, are based on analysis of primary tumours. While analysis of primary tumours provides a full spectrum of genetic

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abnormalities as a whole, it suffers from several disadvantages. First of all the majority of epithelial tumours are highly contaminated with stromal and reactive cells along with infiltrating lymphocytes, which in turn mask allelic losses of some chromosomal regions and, therefore, provide an underestimate of molecular-genetic changes in tumour cells. Secondly the limited amount of tissue available from surgical specimens precludes many studies, which require live cultures for a period of time. Lastly primary cultures are unsuitable for detailed *in vitro* studies. We have made a concentrated effort to develop permanent cell lines from both primary and metastatic colorectal cancer for various biochemical, pharmacological and gene transfer studies, and for identification of tumour suppressor genes associated with multistep colorectal tumorigenesis.

In this study, we present cytogenetic and molecular analyses of 20 newly established colorectal cancer cell lines, with particular emphasis on similarities between chromosomal aberrations and allelic deletion of chromosomal regions 1p, 5q, 17 and 18q, which are believed to be the most common recurrent abnormalities in this particular cancer type. Preliminary results of an attempt to identify chromosomal sites or candidate genes associated with advanced-stage disease and poor prognosis are also presented. These cell lines derived from patient's tumours with known clinical and genetic background are expected to serve as an invaluable resource of material for various future studies.

Material and methods

Cytogenetic analysis: Cell cultures and chromosome preparations were obtained according to methods described earlier (Jhanwar *et al.* 1994). Briefly, single-cell suspension was obtained following enzymatic disaggregation and cells were washed twice with RPMI 1640 growth medium and then transferred to culture flasks containing RPMI 1640, 15% foetal bovine serum, 1% glutamine, 5 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml sodium selenite, 5 μg/ml fungizone, 50 μg/ml streptomycin and 50 U/ml penicillin.

Flasks were incubated at 37°C in an atmosphere of 5% CO_2. The growth medium was changed after 24 hours and unattached cells removed. For permanent cell lines, normal fibroblasts were periodically removed until a pure population of tumour cells was obtained and tumour cells passed through a crisis period, which is normally at about passages 20 or more. *In vitro* cell growth was observed with an inverted phase-contrast microscope and chromosome preparations were obtained following methods routinely employed in the laboratory. Chromosomes were analysed using either quinacrine or Giemsa banding as previously described (Jhanwar *et al.* 1994). Detection of either the same structural abnormality and nonrandom gain of a chromosome in two or more cells or nonrandom loss of a chromosome in three or more cells is considered a clonal abnormality. In addition, a relative deficiency or excess of specific chromosomes, as defined by loss or gain respectively of any given chromosome in relation to the expected ploidy level of the abnormal clone, is also scored to assess nonrandom involvement of specific chromosomes in numerical aberrations (Mitelman 1995). For example, less than three copies of a chromosome in a triploid clone is scored as a loss, whereas more than three copies is scored as a gain. Similarly, a missing chromosomal segment due to an unbalanced translocation is also scored as a partial loss in relation to the expected ploidy level of the clone.

Loss of heterozygosity and allelic deletion study: DNA isolation, measurements, Southern blotting, hybridization and autoradiography were performed according to the methods perviously followed in the laboratory (Presti *et al.* 1991).

Autoradiograms were then analysed and scored for loss of heterozygosity (LOH) in cell lines with matched normal-tissue DNA used as the control. Cell lines that appeared to have clearly lost one allele on the autoradiogram were scored as a loss. Only cell lines with a matched heterozygous normal DNA for the restriction fragment length polymorphism (RFLP) probes were scored for losses, while those with homozygous normal DNA were scored as uninformative. Heterozygous cancers found to have no loss by inspection or densitometry were scored as retained alleles, demonstrating no loss of allele for that segment of the chromosome. Scoring of the autoradiograms was performed independently without knowledge of the cytogenetic results.

Northern analysis of mRNA expression: RNA from the cell lines was extracted using a single-step acid guanidinium thiocyanate phenol chloroform extraction method. The integrity of the RNA was determined by gel electrophoresis (1% agarose) for presence and intensity of the ribosomal RNA bands. Northern blot analysis was performed using 15 mg of total cellular RNA (as determined by absorption at 260 nm) using random-primer-labelled cDNA probes by standard methods. The filter was washed in 0.25% standard sodium citrate – 0.1% sodium dodecyl sulphate at 60°C for 15 min. To verify that there was no significant degradation of RNA, the nitrocellulose filter probed with cDNA was stripped of label by adding boiling water for 30 min and the filter was then autoradiographed overnight to verify that no radioactivity was present; the filter was then rehybridized with a probe for GAPDH.

Immunohistochemistry: Immunohistochemistry was performed by the method described previously by Cordon-Cardo (1994). Two mouse monoclonal antibodies were used. Anti-p53 antibody Pab1801 (Ab-2; Oncogene

Science) recognizes both wild-type and mutant human p53 proteins. The DO7 antibody recognizes mutant p53 proteins. Avidin–biotin peroxidase was performed on cells fixed with cold methanol:acetone. Cells were incubated with 10% normal horse serum (Organon Technika, Westchester) and then incubated with diluted primary antibody. After extensive washing, cells were incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame) and avidin–biotin peroxidase complex (Vector Laboratories). Diaminobenzidine was used as the final chromogen and haematoxylin was used as the nuclear counterstain.

Results

Morphologic, cytogenetic and molecular characterization of colorectal cancer cell lines

The morphology of tumour cells from primary cultures and corresponding cells from established cell lines is strikingly similar (figure 1). As shown in the figure, abundance of tumour cells was noted in both, but stromal cells were present only in primary cultures. Clonal cytogenetic abnormalities detected in cell lines are summarized in table 1. It is clear from these data that recurrent abnormalities of chromosomes 1, 5, 17 and 18 are the most common abnormalities detected in cell lines, which correlates well with abnormalities reported earlier in corresponding primary tumours (Gerdes *et al.* 1995). It is important to point out that all metaphases examined from cell lines were clonally abnormal, whereas primary tumours had a mixture of normal and clonally abnormal metaphases. A representative karyotype from the cell line #19 is presented in figure 2 to show recurrent abnormalities detected in colorectal cancer.

In the 20 cell lines studied, recurrent abnormalities revealed deletions, relative deficiency or rearrangements involving chromosomes 1p (70%), 5q (55%),17p (80%), 17q (30%) and 18q (65%). In addition, abnormalities of chromosomes 7q (25%) and 8p (55%) were also detected. In fact, recurrent abnormalities of these six chromosomes have been previously reported in colorectal cancer by several investigators with increasing details of the nature of abnormalities at both cytogenetic and molecular levels (reviewed in Fearon and Vogelstein 1990; Jhanwar and Gerdes 1994). Although a close correlation was observed between cytogenetic abnormalities and allelic deletions of specific regions of chromosomes 1p, 5q, 17p, 17q and 18q, allelic deletions of chromosomal regions 5q and 18q were identified by RFLP analysis in a relatively lower proportion of cell lines compared to cytogenetic abnormalities, which could be due to a limited number of RFLP probes used for the molecular analysis. Regardless of these minor differences, the allelic deletions of chromosomes 1p (72%), 5q (42%), 17p (89%), 17q (76%) and 18q (50%) were detected in the cell lines studied. In fact, combined use of conventional cytogenetics and RFLP analysis not only identified genetic alterations in approximately 91% of the cell lines studied, but revealed a novel mechanism

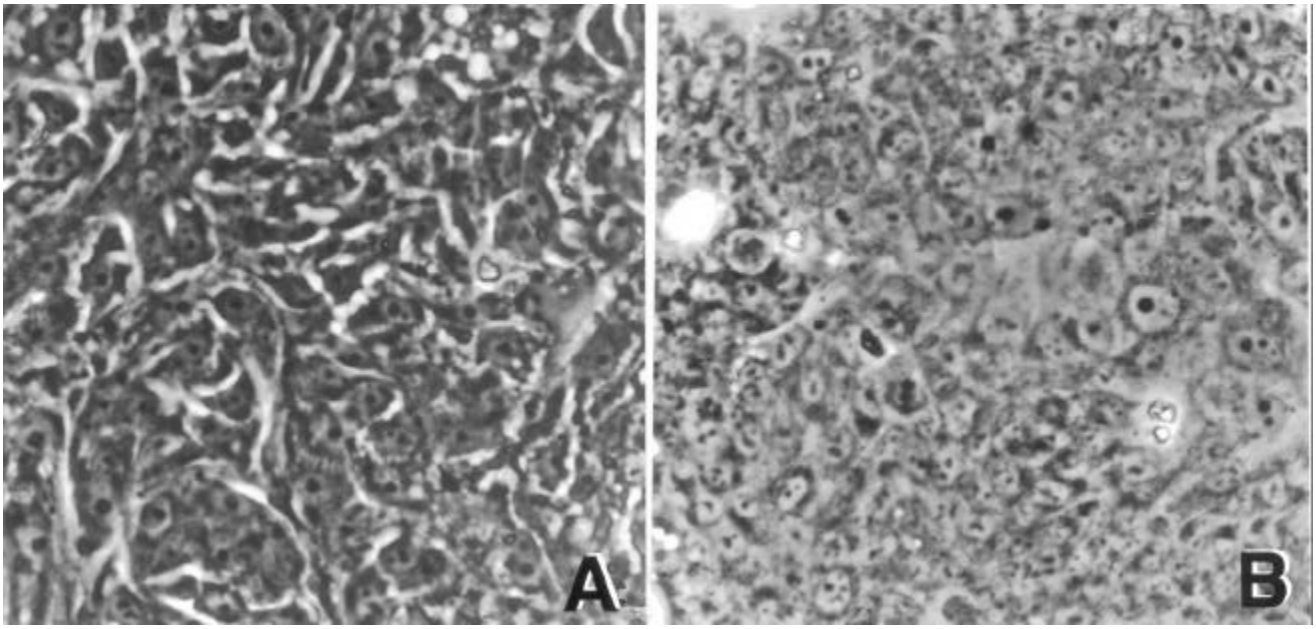

Figure 1. Representative areas from monolayer cells of primary culture (A) and established cell line (B) from the same patient's tumour to show similarity in morphologic features of tumour cells. Note the presence of stromal cells in the primary culture.

Table 1. Summary of clonal cytogenetic abnormalities in colorectal cancer cell lines.

Cell line number	Karyotype
CCCL-2	44–48,X–X,+der(1)t(1;7)(p13;p11.2),+2,+3,del(6)(q21q23),der(8)t(8;22)(p21;q11.2), –10,i(11)(q10),add(11)(p15),i(13)(q10),add(16)(q24),–18,–19,–21,22,+mar1–mar3
CCCL-3	74–76,XY,der(X)t(X :X)(p11;q13),<3n±>,i(1)(q10),–6,+9×2,+13,+15,+19×2,+20
CCCL-4	74–76,<3n±>XX,–X,del(1)(p22p32),+3,+7,+9,i(11)(q10),+13,+der(14)t(14;17) (p11;q11), –18, –19,+20,+mar1
CCCL-5	78–83,<3n±>,XXX,–1,+del(1)(p13p32),add(2)(q44),add(4)(q35),del(5)(q11q31)×2, +I(5)(p10)×2,+del(6)(q15),+del(7)(q22q32),+del(9)(p11),+11,+12,+del(13)(q12q14), +15,add(17)(p11) ×2,+18,–19×2,+20,+21,+22,+dmins
CCCL-6	69–70,XX,i(X)(q10),der(1)t(1;4)(p22;q21),add(2)(q37),–4,del(4)(q21q31),del(5)(p15), –8,del(8)(p21),+11,add(13)(q34),–14,–15,der(16)t(3;16)(q22;q21),add(17)(p11),–18,+18, –20,+22,+mar1,+mar2
CCCL-7	44,X,del(X)(q24),del(5)(q15q31),i(9)(q10),–15, der(15)t(15;17)(p13;q11),–22/87–92, idem×2
CCCL-8	55–56,X,add(X)(p11),–2,+del(13)(p14),–4,+add(5)(q35),–6,add(6)(q25),+12, der(14)t(14;22)(p11;q11),–15,+16,del(17)(p13),+der(18)t(13;18)(q13;q21)×2,del(22)(q13), +ring,+mar1,+mar2,+mar3,+mar4
CCCL-9	51,XY,+5,–8,+13×2,del(17)(q11.2),del(18)(q21),+mar1
CCCL-10	69–79, <3n±>,XXX,+X,–1,+del(1)(p22)×2,–4,+5×2,–6,–7,+del(7)(q32),–8×2,add(9)(p13), add(12)(p13),–13,der(14)t(3;14)(q12;p13),–15,del(17)(p11)×2,–18×2,+2,–21,–22,+mar1
CCCL-11	60–65,XXX,<3n±>,der(1)t(1;4)(p22;q21),–4,–7,+del(7)(q22q32)×2,i(7)(p10),+i(8)(q10),–11, –13,–14,i(17)(q10),–18,+20
CCCL-13	80–82, <3n±>,XXX,+1,del(3)(p21p25),+5,+i(5)(p10),+del(11)(p11.2),+del(11)(q23),+14, +16,del(17)(p11),–18,+19,+20,–22,+mar1
CCCL-14	81–85, <3n±>,XX,–Y,–1,+add(1)(p32)×2,+2,+3,–4,+add(4)(q35)×2,–5,+del(5)(q13q22), +add(5)(p11),+6,+7,+8,+add(9)(q34)×2,–10,+del(10)(p11)×2,+11,–12,+t(12;15)(p13;q15), –15,+16,+del(17)(p11),–20×2,+1–3mars,–22
CCCL-15	52–62,XY,+X,del(1)(p22)×2,+add(5)(q22),+del(12)(p12),–17,–18,+mar1–3
CCCL-16	58–63, <3n±>,XX,–X,der(1)del(p22p32)ins(q22),–2,–4,del(5)(q13q31),–6,der(7)t(7;7)(q32;p15), –8,–9,–11,add(12)(q24),–13×2,–14,–15,–17,–18,–19,+20,–21,–22,+mar1,+mar2
CCCL-18	47,X,–X,der(1)t(1;1)(p32;q21),–8,i(8)(q10),+12,–15,+der(17)t(3;17)(q12;p11)
CCCL-19	69–71, <3n±>,XX,–X,–1,–6,+7,–8,–13,–14,+16,–17×3,+i(17)(q10)×2,+19,+20×2,+21,+mar2×2
CCCL-20	47,XX,+13
CCCL-21	73–79,<3n±>,XXY,+der(3)t(3;10)(q12;11.2),add(4)(q35),+i(5)(p10)×2,+6,+7×2,del(8)(p21), +add(11),(q23)×3,+add(12)(p13),–13×2,der(13)t(13;22)(p11;q11),+der(14)t(3;14)(q12;p11), +der(14)t(14;21)(p11;q11)×2,add(15)(p11),der(15)t(15;18)(p11;q11.2),i(17)(q10)×2, –18,+19,+20×2,–21×3,+3–5 fragments
CCCL-22	42–44,XX,del(1)(22),del(8)(p21)i(11)(q10),–17,del(18)(q21),–21,–22
CCCL-23	60–65,XY,del(1)(p22),–3,–4,add(4)(q31),–5,add(6)(q23)×2,+7,+I(7)(q10),–8, der(8)t(8;22)(q11.2p11)×2,–9,del(9)(p13),–10,+11,add(12)(p13),–13,+14×2, der(15)t(10;15)(11.2;11)×2,–16,–17,–18,–21,–22,+mar1,+mar2,+mar3,+random markers

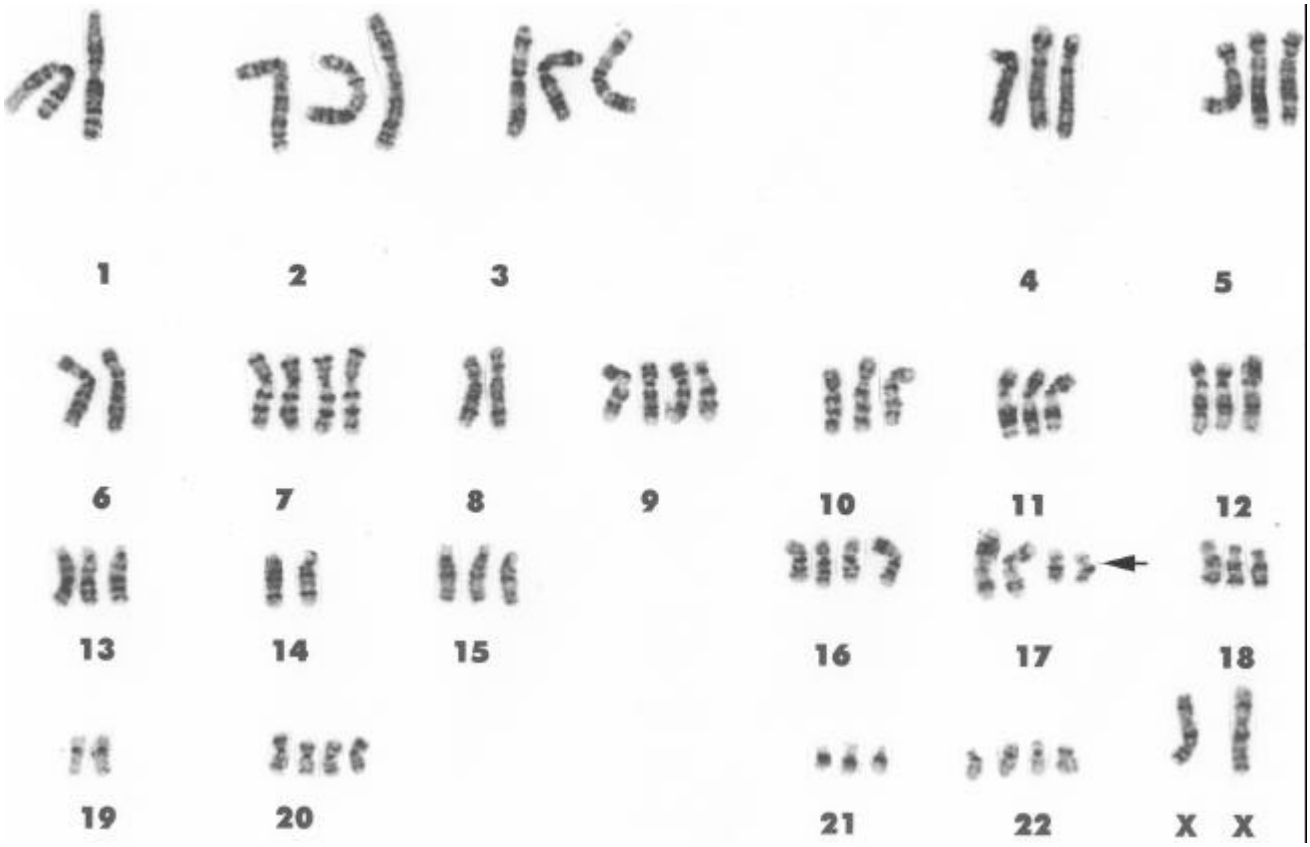

Figure 2. A representative G-banded karyotype from the cell line #19 to show recurrent abnormalities frequently detected in colorectal tumour cell lines. This cell line showed recurrent abnormalities of chromosomes 1, 5, 17 and 18 on the basis of combined results of cytogenetics and RFLP analysis. A balanced translocation t(9p;16p) was seen as a nonclonal abnormality. Also, note that the der(17) initially considered as a marker chromosome of unknown origin based on G-banding alone was subsequently identified as a der(17) based on chromosome-specific painting analysis.

by which recessive mutations are allowed to be expressed at a molecular level by loss of a whole chromosome followed by duplication or triplication of the same chromosome (uniparental disomy or trisomy).

To identify the smallest region of overlap (SRO) on chromosome 1p, we have reexamined the extent of cytogenetic deletions reported earlier (Gerdes *et al.* 1995) and combined it with the additional data generated from the cell lines. On the basis of the combined data, we were able to define three overlapping SROs at 1p36, 1p32–35, and 1p22 in a significant number of cases (figure 3). As an initial approach to define the molecular nature of deletion and identify sites of candidate genes located at 1p, RFLP analysis was performed using a total of five polymorphic probes. The results of such an analysis are presented in table 2 and representative autoradiograms to show LOH are shown in figure 3. As summarized in table 2, 11 (68%) of the 16 informative cell lines showed LOH for markers located at 1p22–32.2, whereas only six (42%) of the 14 informative cell lines lost at least one of the alleles located at 1p36.1–34.3. Although only a limited number of polymorphic probes were used for analysis in this study, a detailed study using a series of probes for 1p localized SRO to 1p35–ter (Leister *et al*. 1990; Bomme *et al*. 1998). Similarly, by using microsatellite markers located at the 1p22 region, we recently identified a region of deletion between D1S1618 and D1S2889 (unpublished results). These results clearly indicate that the gene(s) located at 1p22 and 1p35–36.1 may be critical in the multistep process of tumorigenesis in colorectal cancer. It is also interesting to note that allelic deletion of markers at 1p22 is also frequently observed in other tumour types such as malignant mesothelioma (Lee *et al*. 1996) and various other solid tumours (reviewed in Schwab *et al.* 1996), indicating that tumour suppressor gene(s) located at 1p are not specific to a tumour type. These observations are consistent with the argument that allelic losses from chromosome 1p, which include critical gene(s), may be associated with the progression and metastasis of cancers. The nature of genes located at the sites frequently deleted is, however, not known.

To identify gene(s) on chromosome 17 that confer more aggressive phenotypes, and may be associated with

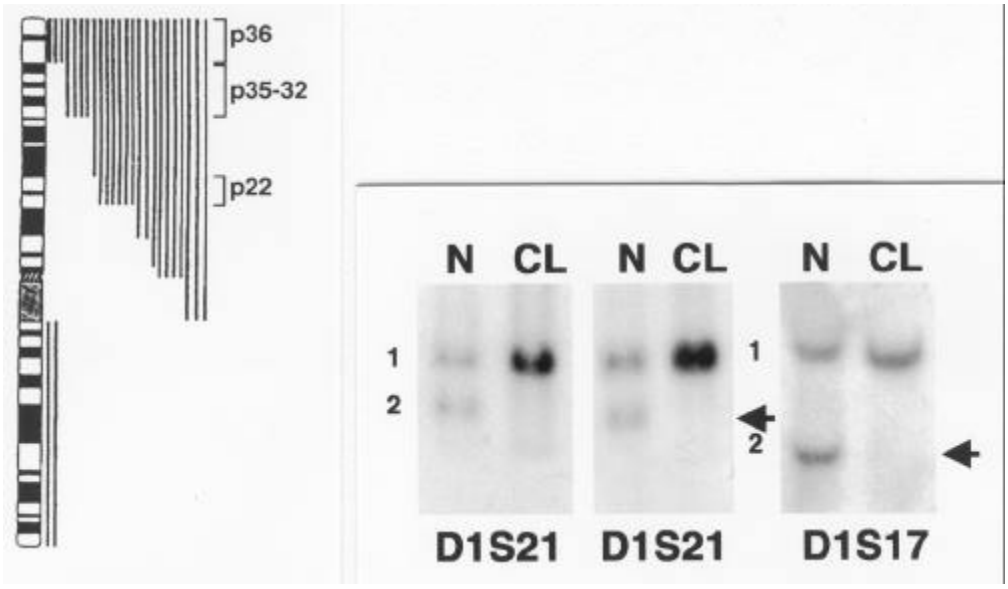

Figure 3. Left panel: An idiogram for chromosome 1 to show the extent of cytogenetic deletions based on abnormality detected in primary tumours and cell lines. Three overlapping deletions, namely 1p36 in 92%, 1p35–32 in 80% and 1p22 in 65% of the tumours and cell lines, are identified. Right panel: A representative Southern blot to show LOH (arrow) using polymorphic markers located at 1p22 in cell lines 15 and 22 and 1p32.2 in cell line 11 (right).

Table 2. Allelic deletion in 1p in colorectal cancer cell lines.

Cell line #	DIS21 (1p22)	DIS17 (1p32.2-31.3)	DIS57 (1p34.3)	DIS80 (1p36-p35	DIS63 (1p36.1)
2	HOM	HET	HET	HOM	ND
3	LOH	HET	HET	LOH	ND
4	HOM	HOM	HOM	HET	ND
5	HOM	LOH	HOM	HET	HET
6	ND	LOH	ND	ND	HOM
7	ND	HOM	HOM	HET	ND
8	ND	LOH	HOM	HET	HET
9	HOM	HET	HOM	HET	ND
10	HOM	LOH	HOM	HOM	ND
11	HOM	HOM	HOM	HOM	ND
13	HOM	LOH	HET	HOM	HOM
14	HET	HOM	HET	LOH	HOM
15	LOH	LOH	ND	ND	ND
16	HET	HET	HOM	LOH	ND
18	ND	LOH	LOH	HOM	ND
19	LOH	HET	HOM	HET	HOM
20	LOH	HET	HOM	LOH	ND
21	HET	HET	HOM	HOM	ND
22	LOH	LOH	LOH	LOH	HOM
23	HOM	HOM	HOM	HOM	ND

HOM, Homozygous; HET, heterozygous; LOH, loss of heterozygosity; ND, not done.
DIS21, DIS17, DIS57, DIS80, DIS63 are RFLP probes.

metastasis, we performed RFLP analysis using several probes including p53 and nm23, Northern blotting to examine for p53 and nm23 mRNA, and immunohistochemical staining (mutant p53 protein). RFLP analysis of the cell lines revealed a very interesting pattern of LOH; several of the cell lines (cell lines 8, 9, 10, 11, 16, 19, 21 and 22) showed LOH for markers located on both arms of chromosome 17 (table 3), indicating a possible loss of the entire chromosome. One such example is illustrated below to show that in spite of disomy, trisomy or tetrasomy of chromosome 17, LOH was detected at molecular level. Cell line #19, with

Table 3. Correlation between allelic deletions of chromosome 17, *p53* and *nm23* expression, and mutated p53 protein in colorectal cancer cell lines.

Cell line #	Grade	Stage	PYNZ-22	PEW-503	P53	PEW-301	NM23	PTH-59	P53 mRNA	NM23 mRNA	% POS	SI
2	PD	C	LOH	NA	HOM	HOM	HOM	HET	–	+	0	0
3	WD	C	HET	HOM	HOM	HOM	HET	HOM	+	+	0	0
4	MD	B	LOH	HOM	HOM	LOH	HOM	HOM	+	+	60	2+
5	PD	D	HOM	HOM	HOM	HOM	LOH	HOM	+	+	20	1.2+
6	PD	C	HOM	HOM	HOM	LOH	HOM	LOH	+	–	30	2+
7	PD	C	LOH	NA	REG	HOM	HOM	HOM	REG	+	0	0
8	PD	D	LOH	HOM	LOH	HOM	LOH	HOM	+	–	40	2+
9	MD	D	LOH	HOM	HOM	HOM	HOM	LOH	+	–	80	2+
10	MD	D	LOH	HOM	LOH	LOH	LOH	HOM	+	+	60	2+
11	MD	C	LOH	LOH	HOM	LOH	HOM	HOM	+	+	20	1.2+
13	MD	D	LOH	LOH	HOM	HET	HOM	HOM	–	+	0	0
14	PD	D	HOM	LOH	HOM	HET	HOM	HOM	+	+	20	2+
15	PD	D	HOM	LOH	HOM	HET	HOM	HOM	+	+	30	2+
16	PD	C	HOM	LOH	HOM	LOH	LOH	HOM	+	+	80	2+
18	MD	D	HET	HET	HOM	HET	LOH	LOH	–	–	0	0
19	MD	B	LOH	LOH	HOM	LOH	LOH	LOH	+	+	< 10	1+
20	MD	B	LOH	HOM	HOM	HET	HOM	HOM	+	+	0	0
21	PD	D	HOM	LOH	LOH	HOM	HOM	LOH	–	–	0	0
22	PD	D	HOM	LOH	LOH	LOH	LOH	HOM	+	+	60	2+
23	PD	C	LOH	HOM	HOM	HOM	HET	HET	+	+	25	2+

HOM, Homozygous; HET, heterozygous; LOH, loss of heterozygosity; REG, rearrangement; SI, staining intensity; NA, not available; –, message absent; +, message present.
PD, Poorly differentiated; MD, moderately differentiated; WD, well differentiated.

chromosome constitution 69–71(3n±),XX,–X,–1,–6,+7,–8,–13,–14,+16,–17×3,+i(17)(q10)×2, +19,+20×2, +21,+mar1×2, following RFLP analysis with probes covering both arms of chromosome 17, showed LOH for all the seven markers used (figure 4); no homozygous deletions for any of the markers on chromosome 17p were detected, indicating that (i) 17p material may be present in the unidentified markers, and (ii) two copies of i(17q) may have originated from the same chromosome 17 following a loss of one of the homologues. Chromosome-17-specific painting analysis performed on this specimen identified 17p material on the marker chromosomes (figure 4). A possible underlying mechanism for such a LOH pattern is also presented in figure 4. In addition, RFLP analysis, summarized in table 3, showed allelic losses around the *p53* locus in 16 out of the 20 cell lines (80%), four of which were losses of the *p53* locus itself. Seven cell lines (out of nine informative cases) also showed losses of the *nm23* gene, four of which had concurrent losses of the *p53* locus, while the remaining three were homozygous for *p53*. In addition, five out of seven cell lines with *nm23* deletions were derived from hepatic metastatic tumours, whereas one cell line was obtained from recurrent tumour. Northern blot analysis (figure 5) showed complete loss of *p53* mRNA expression in four out of the 20 cell lines. Of the remaining 16 cell lines one showed aberrant mRNA due to a germline rearrangement of the *p53* gene, whereas in 15 cell lines normal levels of mRNA were present. Normal levels of *nm23* mRNA were detected in 15 cell lines while five cell lines showed complete loss of expression. A normal germline configuration for the *p53* gene was detected in 19 of the 20 cell lines; one cell line showed a rearrangement. Following immunohistochemical staining, nine cell lines showed high levels (25–80%) of mutant p53 protein (figure 6), four cell lines showed intermediate levels (< 20%), and seven cell lines had low or undetectable levels (< 10%) of the protein; of the seven cell lines with low or undetectable levels of protein, four also had complete loss of *p53* mRNA expression. Of the eight cell lines (seven hepatic metastatic and one derived from recurrent tumour) with allelic deletions of chromosome 1p, six (79%) showed concurrent losses of the *nm23* gene (table 4). In addition, two hepatic metastatic cell lines were homozygous and others heterozygous for 1p markers, but had complete absence of *nm23* mRNA expression. Furthermore, five of the cell lines showed a significant amount of mutant p53 protein, four had either very little or complete absence of mutant p53, and the remaining two cell lines showed intermediate levels.

Discussion

While genetic alterations seen in dividing cells in tumours are a true reflection of the abnormalities of the tumour *in vivo*, abundance of normal cells (stromal and

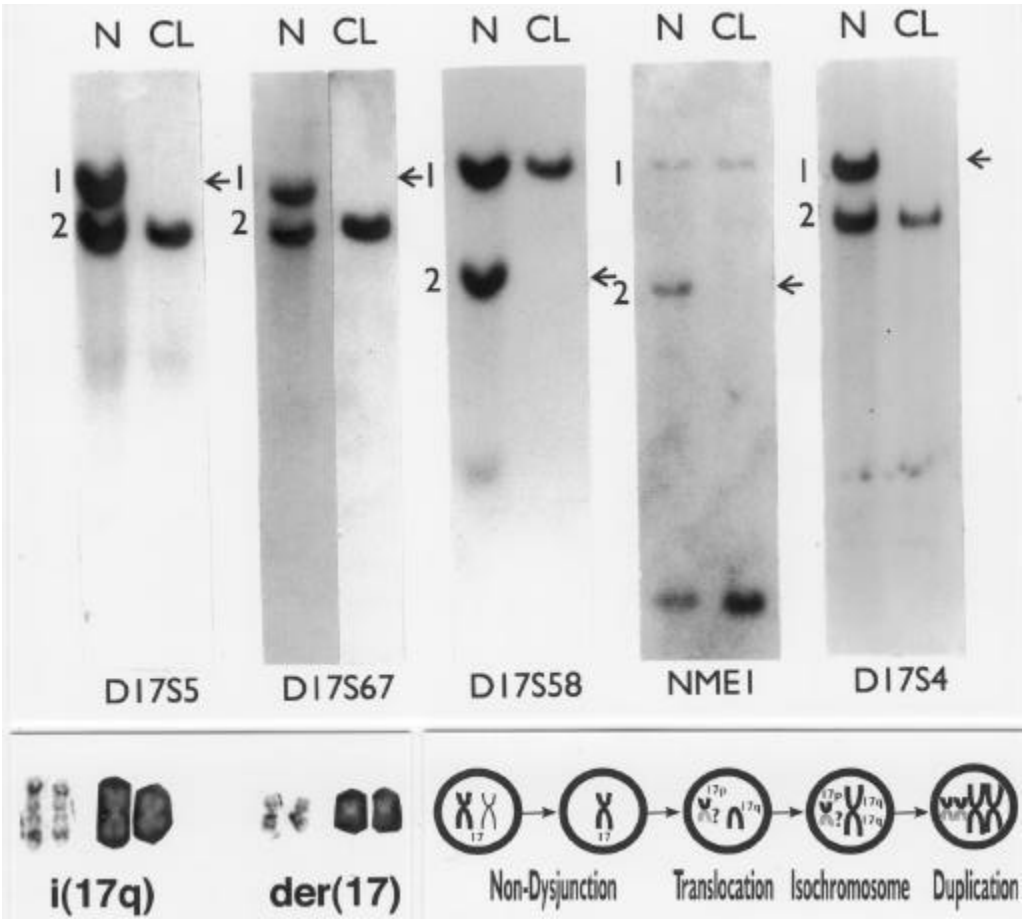

Figure 4. Top panel: RFLP analysis of cell line 19 using polymorphic markers located on both arms of chromosome 17 to show LOH for all the markers studied. Bottom left: Chromosome-specific whole chromosome painting analysis identified two copies of a marker chromosome as two identical copies of der(17), due to unbalanced translocation between chromosome 17p and an unidentified chromosome. Two copies of i(17q) are also clearly identified by the painting analysis. Bottom right: A possible mechanism for loss of heterozygosity of markers from both arms of chromosome 17 and reduplication of the same chromosome resulting in uniparental trisomy or tetrasomy of chromosome is illustrated.

contaminating lymphocytes), invariably present in epithelial cells, most often mask allelic deletions. Established cell lines derived from patients with well-defined clinical and genetic history therefore not only provide an excellent model system for precise molecular characterizations but also serve as reagents for biochemical, molecular and pharmacological studies (Jhanwar 1996).

To employ such cell lines for the studies mentioned above, it is important to establish that the cell lines are phenotypically and genotypically similar to the primary tumours from which they were derived. The results of morphologic, cytogenetic and molecular characterization of established cell lines, with particular emphasis on genetic alterations often associated with colorectal cancer, presented here fulfill this requirement. In fact our combined approach to employ both cytogenetic and molecular methods of analysis to identify genetic alterations in any given cell line not only enhanced our ability to identify recurrent abnormalities associated with multistep tumorigenesis in more than 90% of the colorectal cancer cell lines but also revealed a novel mechanism by which recessive mutations are allowed to be expressed and, therefore, inactivation of tumour suppressor genes via LOH, even in tumours with hyperdiploidy (uniparental disomy, trisomy or tetrasomy of chromosomes).

Since the observation that the gene for familial adenomatous polyposis (FAP) is located at 5q21–22 there has been considerable progress in identification of genetic events associated with colorectal tumorigenesis (Fearon and Vogelstein 1990; Jhanwar and Gerdes 1994). On the basis of such studies, a multistep model of colorectal carcinogenesis has been proposed, according to which *ras* mutations, DNA hypomethylation, *p53*

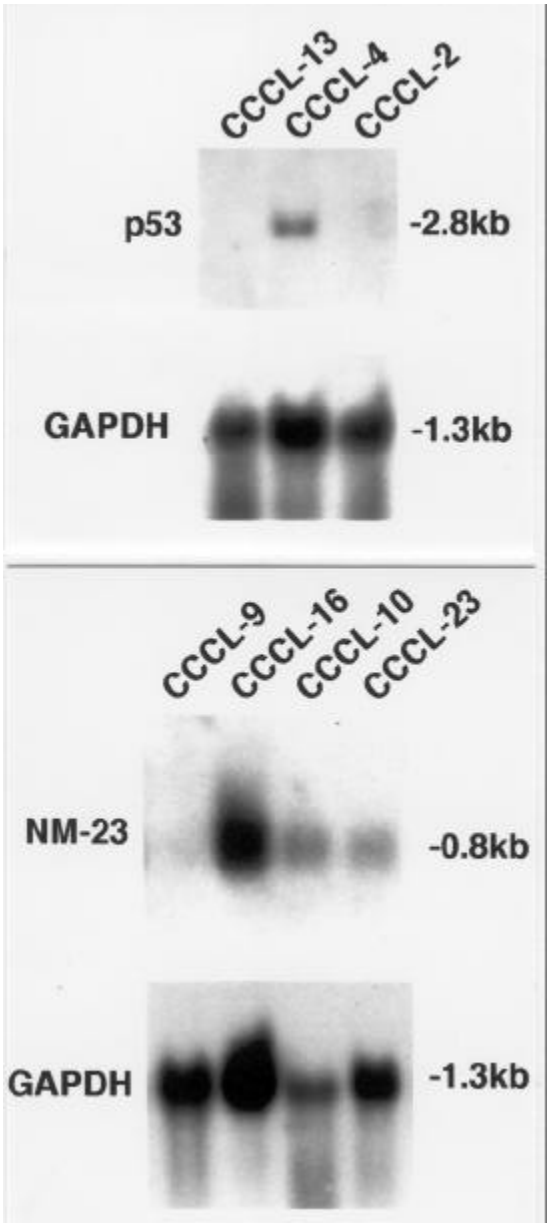

Figure 5. Northern blot analysis of RNA from colorectal cancer cell lines to show complete absence of detectable message in cell lines 2 and 13 for p53 (top) and cell line 9 for nm23 (bottom). The same filters were stripped of label and rehybridized with GAPDH to show expression of GAPDH mRNA, indicating that the mRNA preparation contained approximately equal amounts of intact RNA.

mutation and 5q alterations appear to occur early, while allelic deletions of chromosome arms 17p and 18q usually occur at a later stage of tumorigenesis. Furthermore, it has also been suggested by Jhanwar and Gerdes (1994) that losses of *p53* and *nm23* along with allelic deletions of 1p may be associated with advanced or metastatic disease and therefore may confer poor prognosis. This argument is in agreement with several other

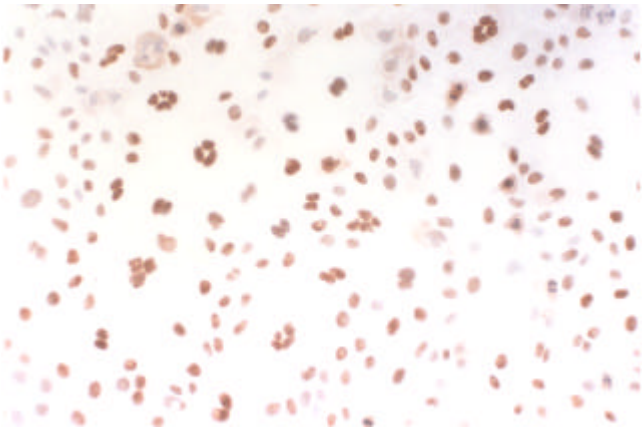

Figure 6. Immunostaining of cells from a colorectal cancer cell line to show mutant p53 protein in approximately 80% of the tumour cells.

studies reported on colorectal cancer (Kern *et al.* 1989; Baker *et al.* 1990; Laurent-Puig *et al.* 1992). From these reports it also became clear that progression of normal colonic epithelial cells to carcinoma involves at least eight mutational events. Although these events need not be the same or occur in a particular sequence, the gradual accumulation of these alterations is necessary for the progression of benign polyps to frank carcinoma and eventually metastasis. The sequence of genetic events associated with initiation, transformation and progression of colorectal cancer, therefore, could be divided into three major categories: (i) a predisposing or tissue-specific genetic change, (ii) alterations in genes associated with transformation, and (iii) alterations associated with progression and metastasis. The genetic alterations associated with predisposition, initiation and progression of normal mucosa cells to invasive carcinoma are now fully established. However, cytogenetic or molecular-genetic alterations associated with metastatic phenotype and those that confer poor response to chemotherapy and hence resistance are not very well defined. It is therefore not unreasonable to assume that identification of genetic alterations in metastatic tumours and recurrent disease may ultimately help in designing new therapies. The results presented here on colorectal cancer cell lines, taken together with studies reported earlier (Cohn *et al.* 1991; Hartsough M. T. and Steeg P. S. 1998), suggest that loss of *nm23* gene or downregulation of its message is correlated with metastasis**.** In addition these results provide sufficient evidence to suggest that functional inactivation of *nm23* and *p53* along with allelic losses at 1p are highly associated with advanced disease and metastasis, regardless of whether the inactivation of these genes is due to a mutation of one allele followed by LOH, loss of mRNA, loss of protein expression, or, as in the case of chromosome

Table 4. Correlation of allelic deletions on chromosomes 1p and 17 with the grade and stage in colorectal cancer cell lines.

Cell line #	Grade	Stage	Allelic deletions, 1p	p53	nm23	mRNA p53	mRNA nm23	Mutant p53 protein (% of cells)
5	PD	D	LOH	HOM	LOH	+	+	20
8	PD	D	LOH	LOH	LOH	+	–	40
9	PD	D	HOM	LOH	HOM	+	–	80
10	MD	D	LOH	LOH	LOH	+	+	60
13	MD	D	LOH	HOM	HOM	–	+	0
14	PD	D	HOM	HOM	HOM	+	+	20
15	PD	D	LOH	HOM	HOM	+	+	30
18	MD	D	LOH	HOM	LOH	–	–	0
19	MD	Rec.	LOH	HOM	LOH	+	+	< 10
21	PD	D	HET	LOH	HOM	–	–	0
22	PD	D	LOH	HOM	LOH	+	+	60

PD, Poorly differentiated; MD, moderately differentiated; Rec., recurrent.
HOM, Homozygous; HET, heterozygous; LOH, loss of heterozygosity.

1p, only LOH. The nature of the critical gene(s) located at 1p, however, is not known yet.

We have employed a candidate-gene approach to identify critical genes associated with 1p22, and recently ruled out the involvement of *BCL10* gene located at 1p22 and known to encode a protein containing an N-terminus caspase recruitment domain homologous to the motif found in several regulatory and effector apoptotic molecules (Apostolou *et al.* 2001). Other candidate genes under investigation using these cell lines include human aflatoxin B1 aldehyde reductase (AFAR) gene located at 1p35–36.1, which is involved in endogenous detoxification of aldehydes and ketones that are known to be causes of tumorigenesis in colon cancer (Praml *et al.* 1998), and yet another gene located at 1p22, which encodes dihydropyrimidine dehydrogenase (DPD), a rate-limiting enzyme of 5-fluorouracil catabolism (Etienne *et al.* 1995; Fischel *et al.* 1995) whose activity is associated with sensitivity of tumour cell lines to 5-fluorouracil.

To assess drug sensitivity, particularly to the antifolate inhibitors of thymidylate synthase, we have used four cell lines, three of which (cell lines 2, 4, 6) are described here. The results of such studies have revealed heterogeneity of response to these drugs, indicating that some of the genetic alterations in these cell lines may contribute to heterogeneous behaviour (Longo *et al.*, in press). However, the specific genetic change associated with such behaviour remains to be seen.

In addition, some of these cell lines are also being used for transfection (gene transfer) with a retroviral construct containing both mutant dihydrofolate reductase (DHFR) and herpes simplex virus thymidine kinase (HSV-TK) or mutant DHFR and cytosine deaminase cDNAs to examine the ability of the drug trimetrexate to enhance expression of these genes. Enhanced expression of these genes is expected to increase the sensitivity of the tumour cell to various chemotherapeutic drugs such as ganciclovir or 5-fluorocytosine respectively (personal communication: J. R. Bertino and colleagues).

Furthermore, in an effort to identify novel tumour suppressor gene(s) located at other sites such as 7q32 (Gerdes *et al.* 1995; Zenklusen *et al.* 1995) and 8p (Fujiwara *et al.* 1993), we have used nine of these colorectal cancer cell lines. Of these, four (44%) showed allelic loss of at least one locus in 7q; two distinct regions of overlapping loss were detected, one at 7q11 (flanked by DNA markers GATA118G10 and D7S2481) and another at 7q31 (between markers D7S796 and D7S490) (data not shown, S. Jhanwar and J. R. Testa). These studies are currently in progress to include more of the cell lines.

The genetic alterations associated with multistep tumorigenesis in colorectal cancer have clinical significance for development of new therapies or prognosis of the disease, but equally important is the fact that the biological significance of these genetic alterations is not fully understood. We strongly believe that the cell lines with various combinations of genetic alterations described here would serve as an important source of material for understanding the biology of tumorigenesis not only in colorectal cancer but other epithelial tumours as well.

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