Isolation and characterisation of a panel of cosmids which allows unequivocal identification of chromosome deletions involving the RB1 gene using fluorescence in situ hybridisation

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Abstract

A series of cosmids covering the majority of the RB1 gene have been isolated from a flow sorted human chromosome 13 specific library. Using fluorescence in situ hybridisation these cosmids were all shown to hybridise to the 13q14 region but not to chromosomes known to carry subband deletions involving the RB1 gene. This panel of cosmids, therefore, can be used objectively for identification of RB1 gene deletions in tumour and normal cells.

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The retinoblastoma predisposition gene has been unequivocally assigned¹ to the 13q14 region by linkage to the esterase D gene (ESD). This localisation had long been suspected^{2,3} because of the observation of constitutional 13q14 deletions in some retinoblastoma (Rb) patients. Approximately 3% of Rb patients carry constitutional 13q14 abnormalities.⁴ Although the majority of these patients have extensive chromosome deletions, which result in a variety of associated congenital abnormalities, a few carry only subband deletions which could only be detected using high resolution chromosome banding techniques. Although severe mental retardation and dysmorphic features are usually associated with patients with large deletions involving 13q14,⁵ which is usually a sufficient indicator of the presence of such a deletion, patients with small, subband deletions may have very few associated phenotypes. We have shown previously that, on occasion, these small deletions may be overlooked using conventional trypsin-Giemsa banding procedures in routine laboratories.⁶ It is also possible that there are constitutional deletions, as yet undetected, which involve only the RB1 gene or even part of it which cannot be detected cytogenetically. We have reported one case, for example, of a partial intragenic deletion of the RB1 gene using conventional restriction fragment length polymorphism analysis.⁷ This deletion was only detected by chance because it involved two of the adjacent polymorphic loci used routinely in linkage analysis. Evidence of small intragenic deletions of the RB1 gene in tumours has also been reported.⁸

In the UK, Rb patients are routinely analysed cytogenetically since the presence of a chromosome deletion means that, in the future,

prenatal screening of subsequent children is possible in these families. With the development of fluorescence in situ hybridisation (FISH) technology, which shows the presence of DNA sequences directly on metaphase chromosomes, the unequivocal existence of deletions of the RB1 gene can be established. If these deletions are restricted to the RB1 gene they will not be associated with congenital abnormalities which might otherwise identify these patients. Furthermore, since deletions do not affect fertility they can be passed on through the generations. In fact, these deletions will also be missed in screening programmes designed to identify mutations by sequencing^{9,10} since only the normal allele will be present in constitutional cells. To this end we have isolated and characterised a series of cosmids covering the 5' and 3' ends of the genomic sequence of the RB1 gene which can be used in FISH analysis of chromosomes from Rb patients.

Materials and methods

ANALYSIS OF COSMIDS

Since plasmid sequences would cross react with the cosmid vector in hybridisation experiments using the gridded filter, the 3.8R and 1.8M probes were isolated by electrophoresis and purified using the 'GeneClean' procedure. The gridded filters, provided by the ICRF Genome Analysis Laboratory, were hybridised in Church buffer overnight with the probe radiolabelled by oligonucleotide extension as described by Feinberg and Vogelstein.¹¹ DNA was prepared from cosmids using standard alkali-lysis procedures¹²; 100 µg/ml RNAase was added and incubated at 37°C for 45 minutes and DNA purified by phenol/chloroform extraction. The cosmid DNA was finally precipitated using two volumes of ethanol and resuspended in approximately 200 µl TE.

FLUORESCENCE IN SITU HYBRIDISATION

Cosmid DNA (approximately 1 µg per reaction) was labelled with biotin-14-dATP (Gibco) using the Bionick kit (Gibco) which introduces biotinylated nucleotides into the cosmid DNA by nick translation with DNAase. The reaction was allowed to proceed for one hour at 16°C followed by one hour at 37°C as described by Pinkel *et al.*¹³ Repetitive sequences in the cosmid were competed out with *Cot*I DNA (Gibco) as described by the manufacturers. Hybridisation was allowed to

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Figure 1 *Hind*III restriction band patterns of cosmids spanning the 3' end of the *RB1* gene.

proceed for 24 to 72 hours in a humidified chamber at 37°C. The slides were then washed three times in 50% formamide/2× SSC followed by three washes in 2× SSC both at 42°C. The slides were then prepared for immunological detection of the biotinylated probe by washing in 4× SSC/0.05%. Chromosome spreads were then viewed using a CCD camera (Photometrics) and the Smartcapture software (Digital Scientific, Cambridge).

Results

Flow sorted chromosomes were used to create a cosmid library (C107) for human chromosome 13, the details and characterisation of which will be presented elsewhere (Nizetic *et al*, submitted). These libraries are gridded on nylon membranes at high density for screening by hybridisation. The human *RB1* cDNA is 4.7 kb long and contains an *Eco*RI site which produces two fragments, 0.9 kb and 3.8 kb long, termed 0.9R and 3.8R respectively.⁸ The 3.8R fragment covers exons 9–27 and behaves

Figure 2 Autoradiograph of *Hind*III digested cosmid DNA probed with 3.8R. Although the 2.1 kb fragment corresponding to exon 27 of *RB1* is present in H0885, new bands are also seen, possibly suggesting internal rearrangement of this cosmid.

as a unique sequence probe although, when hybridised to total genomic DNA digested with *Hind*III for example, produces hybridising bands on the autoradiograph which largely correspond to the 3' end of the gene covering exons 18–27. When we screened the gridded chromosome 13 library with 3.8R, seven individual cosmids were identified and recovered. *Hind*III digests of DNA from these cosmids (fig 1) show that each has restriction fragments in common as well as a few which are unique. When these cosmids were probed with the 3.8R fragment all showed positive hybridisation, although it was clear that only certain bands hybridised in some cosmids compared with others (fig 2). From an analysis of the hybridisation pattern it was possible to construct an overlap map of these cosmids which extends into the 3' untranslated region of the gene and into the large (70 kb) intron¹⁴ between exons 17 and 18 (fig 3). Cosmid H0885 showed an abnormal hybridisation pattern which was not analysed further since the remaining overlapping cosmids proved adequate for the FISH analysis. Curiously, only the exons at the 3' end of the gene were present, possibly because the hybridisation to exons 9–17 is only weak and so were not identified in the primary screen. This is consistent with Southern blot hybridisation experiments where the restriction fragments which hybridise strongly are those encompass-

Figure 3 Physical map of the 3' end of *RB1* showing the relative location of the seven cosmids, 11–16. The solid lines represent regions of the gene confirmed by Southern blotting to be in the particular cosmid and the dotted line indicates the probable extensions.

Table 1 Summary of hybridisation for each of the seven cosmids using the 3.8R probe. The reference number refers to the ICRF reference library database and the cosmid number is the laboratory quick reference code

Cosmid	HindIII fragments				
	Reference No	5.3 kb	9.8 kb	6.2 kb	2.1 kb
RB cos 15	C108 B0252	+	+	+	+
RB cos 16	C108 F0473	+	+	+	+
RB cos 13	C108 C0460	+	+	—	—
RB cos 14	C108 A03202	—	+	+	+
RB cos 12	C108 A0185	+	+	+	+
RB cos 11	C108 C04111	+	+	+	+

Table 2 Summary of PCR results for exons 1 and 2 for each of the five cosmids identified by their ICRF database reference number

	Exon 1	Exon 2
C 108 E135	+	+
C 108 F1154	+	+
C 108 F266	+	+
C 108 G291	+	+
C 108 H170	+	—

Figure 4 FISH analysis of normal metaphase chromosomes (top) using cosmid G291 showing hybridisation to two homologues of chromosome 13 (arrows). The centromeres of chromosome 13 have been identified using an α satellite probe, the signal from which lies above the RB1 specific sequence on each homologue. This α satellite also cross reacts with chromosome 21 and two small acrocentric chromosomes also show hybridisation signal above their centromeres (arrow heads). Chromosome analysis of GOS 115A (bottom) is shown where the two homologues of 13 are identified by strong centromeric staining (closed arrows) but only one homologue shows hybridisation signal in the q14 region. The satellites at the centromeres of both copies of chromosome 21 are also seen (arrowheads).

ing exons 18–27. The absence of exons 9–17 in these cosmids was confirmed using PCR and the oligonucleotides described by Hogg *et al.*¹⁵ Details of the cosmids from the 3' end of the gene, together with their reference library numbers, are given in table 1.

The exons of the RB1 gene cluster in three distinct groups separated by two large introns.¹⁶ We were unable to identify cosmids covering the central part of the gene. The 0.9R fragment covers the first eight exons of RB1¹⁷ and contains repetitive elements, which makes it difficult to use directly in hybridisation experiments. In order to isolate cosmids from the 5' end of the gene we used a unique sequence DNA probe, M1.8,¹⁸ located in intron 2 of RB1. From this screen five cosmids were isolated all of which were shown to contain exons 1 and 2 using PCR (table 2) and their location within the RB1 gene is shown in fig 3.

The seven cosmids from the 3' end of the gene and the five cosmids from the 5' end were used in FISH analysis using metaphase chromosomes from normal persons and they all produced a positive signal exclusively in the q14 region of chromosome 13 (fig 4). This strongly suggests that none of these cosmids is chimeric. To determine whether these cosmids could be used to identify chromosome deletions, metaphase chromosomes were prepared from a lymphoblastoid cell line, GOS 115A, derived from the patient discussed by Cowell *et al.*⁶ with a subband deletion of 13q14. Hybridisation was only observed on one chromosome 13 homologue in each metaphase spread (fig 4).

During the course of this analysis two patients were referred to us for ESD analysis who carried deletions involving 13q14. These patients were karyotyped because they showed dysmorphic features and developmental delay. One patient showed del(13)(q12q14.1) the other del(13)(q14q22). In situ hybridisation showed that only one of the chromosome 13 homologues showed hybridisation with the RB1 cosmids in both cases, confirming that the RB1 gene was included in the deletion. Since this diagnosis the child with the del(13)(q12q14.1) has developed tumours but the other patient has not yet, although she is still only 5 months old. We have noted in the past⁴ that the majority of deletion 13 cases tend to have a later onset of disease than persons carrying the hereditary predisposition owing to point mutations in the RB1 gene. Clearly the del(13)(q14q22) patient must still be screened ophthalmologically for the foreseeable future.

Discussion

The series of cosmids described here can clearly be used to identify Rb patients with constitutional deletions of the RB1 gene. Until now, deletions have traditionally been identified either through cytogenetic G banding analysis, which can be very subjective when small deletions are involved, or by the demonstration that patients have reduced red cell ESD levels.^{4,19} The ESD gene, which lies proximal to RB1,²⁰ is at least 200 kb away²¹ and possibly even further. Thus, deletions of RB1 which do not extend as

far as ESD would not be detected by ESD quantitation. Deletions in Rb patients seem to fall into two classes⁴; those with 13q12-q14 deletions and those where the proximal breakpoint lies in 13q14 and the deletion extends distally. From this observation alone it cannot be determined whether RB1 is lost as a result of the deletion. Because these patients usually have mental retardation many of the deletions are identified in the patient before tumours have developed. At this stage the question, therefore, is whether the 13q12-q14 deletions extend as far as RB1 or, when the proximal breakpoint is in 13q14, is it centromeric to RB1?

The RB1 gene lies on the chromosome with the 5' end towards the centromere.²² Thus, patients presenting with deletions involving proximal parts of the chromosome should be analysed with the M1.8 cosmids and patients with deletions extending distally should be analysed with the 3' cosmids. Failure of any of these cosmids to hybridise means that the patient has lost part, or all, of the RB1 gene and is therefore "at risk" of tumorigenesis and should therefore undergo repeated ophthalmological analysis. Clearly, in routine applications of this technology, representatives from most of the cosmids should be used and a sufficient number of metaphase spreads analysed to confirm that a deletion exists. This type of analysis should supersede ESD quantitation studies which are time consuming to set up especially for small sample numbers, particularly since quality controls must be run before each new set of samples are analysed.¹⁹ The other advantage of FISH analysis over ESD quantification is that it will detect deletions which do not extend to the ESD locus. One such example was reported by Cowell *et al.*²³ where the deletion breakpoint lay between ESD and RB1.²⁰ The other advantage of FISH is that the subjectivity associated with metaphase chromosome analysis is removed, particularly when the deletions are small and confined to the 13q14.3 region. In future, therefore, RB1 deletions can be detected by technicians not trained in G banding analysis since, by using the α satellite probe which recognises chromosomes 13 and 21, it is relatively easy to identify the two homologues of chromosome 13 as we have shown. A recent report by Kallioniemi *et al.*²⁴ used 14 phage lambda clones covering RB1 and FISH analysis which could be used to identify RB1 deletions. We feel that cosmids have advantages over phage in that fewer clones are needed to span the gene and it is far simpler to prepare DNA from them. It is unlikely that FISH will detect small intragenic deletions, however, since the cosmids or lambda clones will span the deletion and hybridisation may not be compromised.

The RB1 gene is also deleted and rearranged in a variety of other tumours including osteosarcoma,⁸ breast tumours,²⁵ small cell lung cancer,²⁶ and B cell leukaemia.²⁷ In the latter case particularly, chromosome morphology from tumour cells may not be of sufficient quality to identify small deletions but the panel of cosmids described here will clearly assist in the evaluation of allele loss in those tumours

where conventional cytogenetics may have been equivocal.

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