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Original Paper

Allele Loss on Chromosome 11q and Microsatellite Instability in Malignant Melanoma

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Loss of heterozygosity (allele loss, LOH) occurs frequently on the long arm of chromosome 11 in several types of cancer. We analysed 32 melanomas (almost all metastatic lesions) for allele loss at eight loci along the length of chromosome 11 (*ptel-D11S922-D11S899-D11S1324-D11S1313-D11S901-NCAM-D11S29-D11S968-qtel*). The highest frequency of loss (38%) was at *D11S29* (11q23.3). Of 13 melanomas which had lost an allele at one or more loci, all but one showed LOH at either *D11S29* or *NCAM* (11q22). The region between these two loci is the most likely location of any tumour suppressor gene. Low frequencies of LOH occurred on 11p and there was little evidence for tumour suppressor loci outside the 11cen–q23.3 region. Unusually for melanomas, widespread microsatellite instability, with slippage of several repeat units, was observed in two of 32 tumours studied (and four other tumours showed new microsatellite alleles that differed by just one repeat unit from their normal counterparts). However, no mutations of the mismatch repair genes *hMSH2* and *hMLH1* were detected in these two tumours, and the observed replication errors may result from mutations in other genes involved in mismatch repair or DNA replication. LOH on 11q and replication errors appear to comprise part of the genetic pathways of several tumour types, including melanomas.

Key words: melanoma, 11q, microsatellite instability, HNPCC, LOH
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INTRODUCTION

FREQUENT LOSS of heterozygosity (LOH, allele loss) in tumours is taken to represent the inactivation of a nearby tumour suppressor gene. LOH may result from deletions, chromosomal non-disjunction, mitotic recombination or gene conversion. In melanomas, allele loss often occurs on chromosomes 9p [1, 2], 10q [3], 6q [4] and 11q [5]. In the last of these regions, a frequency of allele loss of 58% has been reported using restriction fragment length polymorphism (RFLP) analysis at the *D11S29* locus. Subsequently, a somewhat lower frequency of LOH at loci on 11q (26%) was reported by Walker and associates [6] using a microsatellite marker at the *D11S912* locus. 11q LOH appeared to occur as a late event and was found only in metastatic melanomas. Healy and associates [7] found only a 15% frequency of LOH on 11q at *D11S910*, but their melanomas were almost all primary cutaneous lesions. Herbst and colleagues [8] found allele loss at the *APOC3* locus (11q23) in 30% of melanomas, more frequently in secondary lesions. Cytogenetic evidence has also supported a rôle for chromosome 11q in melanoma pathogenesis [9–12].

Frequent LOH occurs on chromosome 11q in several other types of cancer, including breast cancers [13–16], cervical cancers [17–18] and ovarian tumours [19]. It is unknown whether the same loci are involved in all these tumours, but a locus between 11q22-qter may provide a selective advantage when lost in many types of tumour. It is interesting in this respect that Tomlinson and colleagues [16] found an association between 11q allele loss and the presence of metastatic disease in breast cancer, similar to the association between 11q LOH and the derivation of tumour material from a metastasis found in melanomas by Walker and coworkers [6].

In addition to LOH, microsatellite instability—a form of replication error (RER)—occurs frequently in tumours. It results from slippage in the number of repeat units at microsatellite loci. Microsatellite instability appears to be rare in melanomas, and when it does occur, slippage of only one repeat unit is usual [6, 20]. This compares with slippage of several

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repeat units that is commonly seen, for example, in some colon cancers, especially those of the hereditary non-polyposis colon cancer (HNPCC) syndrome. Microsatellite instability in colorectal cancers is often caused by mutations of genes that are involved in repairing mismatched bases in DNA.

In this study, the frequency of allele loss in malignant melanoma at the *D11S29* locus was determined using a more informative marker than that used previously [5]. It was unknown whether the high frequency of LOH at *D11S29* primarily affects loci close to this marker or is a secondary effect of allele loss at loci some distance away from *D11S29*, perhaps even on chromosome 11p. We therefore refined the region of greatest allele loss on chromosome 11 by studying seven other microsatellite markers mapping along the length of the chromosome (Figure 1). All eight markers were also used to determine the frequency of microsatellite instability in the tumours analysed. In cases with widespread microsatellite instability, mutations were searched for at the *hMLH1* and *hMSH2* loci: these are the genes most commonly mutated in the germ-line and somatically in colorectal cancers with microsatellite instability.

MATERIALS AND METHODS

LOH and RER analysis

DNA was extracted by standard methods from thirty-two cases of sporadic malignant melanoma (primary tumour or metastasis) and normal tissue or peripheral blood as controls. All visible normal tissue was removed from tumours prior to extraction of DNA, but tumours were not microdissected. Paired samples of tumour and normal DNA were genotyped at the following dinucleotide repeat markers on chromosome 11 (Table 1, Figure 1): *ptel-D11S922-D11S899-D11S1324-D11S1313-D11S901-NCAM-D11S29-D11S968-qtel*). PCR reactions were performed using 200 ng of each DNA, whether derived from tumour or normal tissue, in a final volume of 50 μl. Final reaction concentrations of 1× standard PCR buffer (Promega, U.K.), 1.5 mM Mg^{2+}, 0.5 mM dNTPs and 0.4 mM of each specific oligonucleotide primer were used. One unit of Taq polymerase was added per reaction. For all markers except *D11S29* and *NCAM*, amplification was performed using a protocol of 94°C (1 min) × 1, 94°C (1 min)/55°C (1 min) × 35, 72°C (5 min) × 1 [21]. For *D11S29* and *NCAM*, amplification was performed using a protocol of 94°C (1 min) × 1, 94°C (1 min)/55°C (1 min)/72°C (1 min) × 30, 72°C (10 min) × 1 [22]. PCR products were heated to 90°C for 5 min and electrophoresed on a 6% acrylamide sequencing gel (Sequagel, U.K.) under denaturing conditions for 2–4 h. DNA was transferred by blotting on to Hybond N+ membranes (Amersham, U.K.) for 4–16 h. PCR products were detected by the enhanced chemiluminescence technique (Amersham, U.K.), using a randomly elongated oligonucleotide primer as a specific probe for each locus. Products were visualised by exposing membranes to Hyperfilm (Amersham, U.K.) from 1 min to 1 h. Allele loss was scored by eye alone in heterozygous (informative) cases. Hence, whilst analysis by eye may slightly underestimate the frequency of allele loss, only unequivocal cases scored as loss. Extra bands in tumour samples that differed by multiples of 2 base pairs (bp) from their normal counterpart were scored as RERs (replication errors). Allele loss at two microsatellite markers, *D2S123* and *D3S1611* (closely linked to *hMSH2* and *hMLH1*, respectively), was also studied using the method given above in two tumours (cases 5 and 17), which showed widespread microsatellite instability.

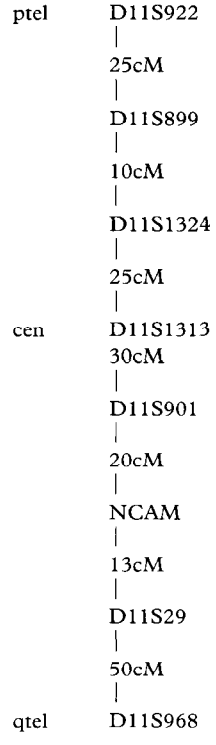

Figure 1. Approximate genetic map of the loci studied.

Single strand conformational polymorphism (SSCP) analysis

Each exon of the *hMSH2* and *hMLH1* genes was amplified individually in specific PCR reactions using the pairs of oligonucleotides and reaction conditions described by Beck and colleagues (details from authors). In general, oligonucleotides were intronic, located 0–40 bp from each intron–exon boundary. PCR products were heated to 90°C for 5 min and electrophoresed at 20 mA for 12 h on a 10% acrylamide:*bis*-acrylamide gel (59:1, with 10% glycerol) at room temperature under non-denaturing conditions. DNA was detected by silver staining gels using standard protocols. SSCP analysis was chosen as the technique for mutation detection because (a) only genomic DNA was available from the tumours and (b) it had previously been used successfully to detect mutations in a number of HNPCC families.

Clinical data

The following clinical data were also known: primary or metastatic tumour origin, and patient age at presentation. Using standard statistical techniques, associations were searched for between the clinical data and allele loss/RERs.

RESULTS

Loss of heterozygosity

A moderately high frequency of allele loss was found at loci mapping to 11q22–q23.3 (Figure 2): nine of 24 (38%) informative cases had lost alleles at the *D11S29* locus and eight of 23 (35%) cases showed LOH at *NCAM* (Figures 2 and 3). Of thirteen melanomas which had lost an allele at one or more loci, all but one showed LOH at either *D11S29* or *NCAM*. The region between these two loci is the most likely location of any tumour suppressor gene; low frequencies of

Table 1. The loci studied

Name	Map position	Repeat unit	Size of alleles (bp)	Heterozygosity
D11S922	11pter	CA	88–138	0.93
D11S899	11p	CA	87–111	0.71
D11S1324	11p	CA	110–128	0.82
D11S1313	11cen	CA	184–204	0.85
D11S901	11q	CA	160–176	0.82
NCAM	11q22–q23	CA	101–119	0.79
D11S29	11q23.3	CA	143–163	0.77
D11S968	11qter	CA	137–155	0.81

LOH occurred on 11p and there was little evidence for tumour suppressor loci outside the 11qcen–q23.3 region. LOH at *D11S29* and *NCAM* was variously caused by events affecting the whole chromosome, part of the long arm, or interstitial regions on chromosome 11. Single mutations could account for most of the allele loss at *D11S29* and *NCAM*: in only two of six doubly informative cases were alleles lost at one locus, but retained at the other. In two cases, alleles were lost at all informative loci along the chromosome, suggesting that non-disjunction of chromosome 11 had occurred. In a further two cases, single alleles were retained, suggesting either false-negative results at the loci scored as 'no LOH', or non-disjunction together with another mutation (such as translocation) affecting the loci without allele loss. Allele loss incompatible with non-disjunction occurred at the centromeric *D11S1313* locus in six cases, suggesting that mitotic recombination or gene conversion was responsible for the LOH in these tumours.

Replication errors

RERs were observed in six of the 32 (19%) melanomas studied. These tumours (7, 9, 13, 15, 26 and 28) showed no LOH. In four of these six cases, the RERs involved just one microsatellite repeat unit, either at one locus (three cases) or at two loci (one case). In two tumours (cases 7 and 15), RERs occurred at five and six loci, respectively (Figure 3). The allelic patterns in cases 7 and 15 resembled microsatellite instability seen in cancers from HNPCC patients and might therefore have resulted from mutations at the *hMSH2*, *hMLH1* or other loci involved in mismatch repair. It was therefore decided to search for mutations at *hMSH2* and *hMLH1* in tumours 7 and 15.

SSCP analysis

In none of the 16 exons of *hMSH2* or the 19 exons of *hMLH1* was any variant SSCP band detected in melanomas 7 and 15. Neither of these cases had lost an allele at *D2S123* or *D3S1611*; microsatellite instability was observed at *D2S123* in tumour 15. These results suggest, therefore, that patients 7 and 15 have neither germ-line nor somatic mutations of the *hMSH2* and *hMLH1* mismatch repair genes. Ideally, other mismatch repair loci, such as *hPMS1* and *hPMS2*, would have been screened for mutations, but the genomic structure of these genes was unknown and no cDNA was available from any of the tumours studied.

Associations with the clinical data

Four tumours studied were of primary cutaneous origin (cases 1, 8, 18 and 28); case 31 was of unknown origin. All other tumours were derived from metastases, although the site of metastasis was unknown for most of these samples. Patients presented at a mean age of 56 years (S.D. 15.4, range 21–82). There was no association between age of presentation and origin of the tumour from primary lesion or metastatsis. The great predominance of secondary (metastatic) tumours in the sample (27/31, 87%) means that associations between allele loss and tumour origin are difficult to determine. However, of 10 tumours with LOH at *D11S29*, all were metastases. One primary melanoma had lost an allele at *NCAM*, but this tumour had retained alleles at all other informative loci; the significance of this isolated region of LOH is therefore doubtful. Apart from this one example of LOH at *NCAM*, no evidence of allele loss was found in any of the primary tumours. Age of presentation was not associated with LOH at *D11S29* and/or *NCAM* ($t = 1.0$, d.f. = 20, $P > 0.3$), or with LOH at any of the loci studied ($t = 0.8$, d.f. = 29, $P > 0.3$). No associations were found between the clinical data and the presence of RERs in the tumours.

DISCUSSION

The frequencies of allele loss detected at loci on chromosome 11q22–q23.3 in this study confirm suggestions that this chromosome may be important in the pathogenesis of malignant melanoma. The highest frequencies of loss were at *D11S29* (38%) and *NCAM* (35%); the most likely location of a tumour suppressor gene is between these loci. The high frequency of LOH (58%, 7 of 12 tumours) that had been found previously at the *D11S29* locus, using an RFLP-based polymorphism with a low information content [5], was borne out by this study. Here, the use of a *D11S29* microsatellite marker with heterozygosity of approximately 0.8 increased the effective sample size to 24 informative tumours. In comparison with the original report of LOH at *D11S29*, the frequencies of LOH detected here are lower, but still raised. There is no significant difference between the results of this study and those obtained previously ($\chi^2 = 0.62$, $P > 0.5$). This study included 12 cases that had previously been studied by Tomlinson and associates [5], but which had been non-informative at the *D11S29* locus using the RFLP-based polymorphism; two cases (6 and 11) which previously showed LOH at *D11S29* also showed LOH with the microsatellite *D11S29* marker used here.

Coarse-scale mapping of allele loss was used to decide whether the locus originally studied (*D11S29*) was the site of maximum LOH, or whether LOH actually occurred more frequently elsewhere on chromosome 11 (Figure 2). Low frequencies of LOH were found on the short arm of chromosome 11 and there is little evidence of a tumour suppressor

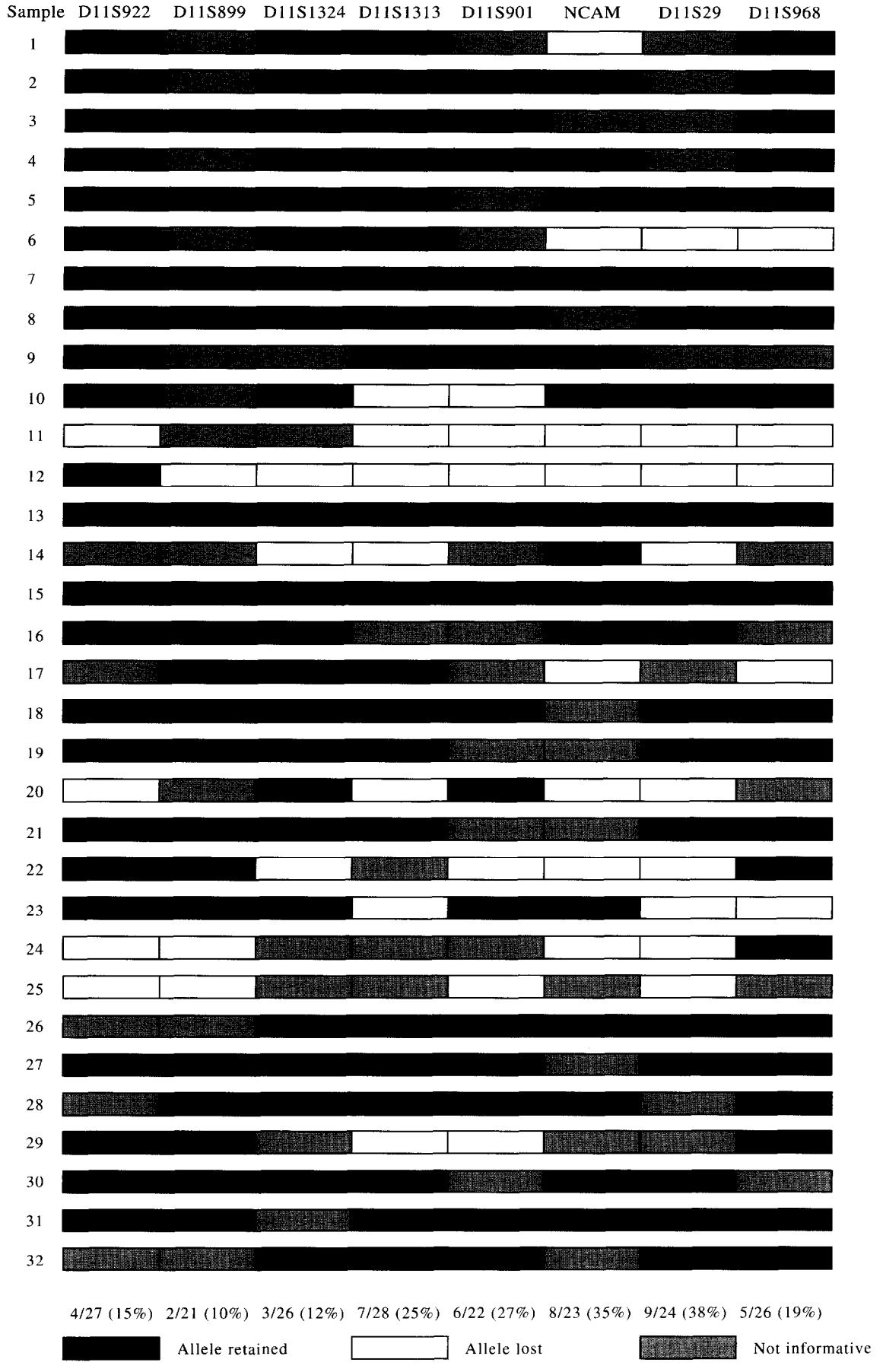

Figure 2. The patterns of allele loss on chromosome 11q. Below each column are shown (no. cases with LOH)/(total no. informative cases at that locus).

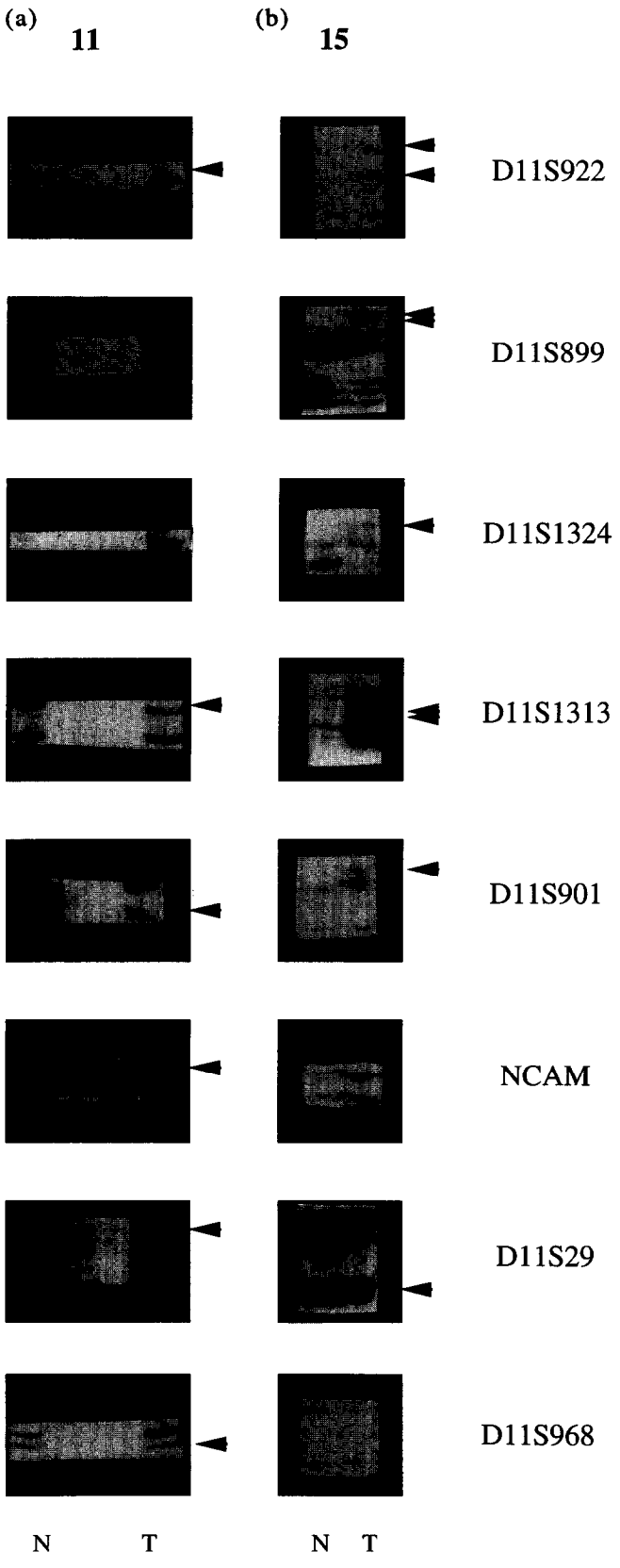

Figure 3. Examples of allele loss and microsatellite instability. Loci are shown in chromosome order. The constitutional (normal, N) alleles at each locus (left) are displayed adjacent to the corresponding tumour (T) alleles (right). (a) Case 11. Alleles are lost at all informative loci (arrowed). This case is not informative at *D11S899* and *D11S1324*. (b) Case 15. Novel tumour alleles suggestive of microsatellite instability (arrowed) are seen at six of the eight loci. The novel allele at *D11S29* is just visible as a subtle shift of one band in the lower tumour allele, accompained by a diminution of intensity in the lowest (stutter) band of the same allele. Instability at *D11S1313* is demonstrated by the lower arrowed novel band in the tumour, and possibly also by the upper arrowed band; note that the lower wild type allele in the tumour is less intense than the upper allele (when a slightly greater intensity of the lower band would be expected from comparison with the normal DNA), suggesting that the novel bands arose from this allele.

gene on 11p. Alleles at the *D11S968* locus (which maps close to 11qter) were lost infrequently. The most likely location of any tumour suppressor gene is therefore somewhere between *D11S1324* and *D11S968*. Although the region around *D11S29* and *NCAM* has the maximum frequency of LOH and is our favoured location for a tumour-suppressor gene, relatively high frequencies of loss were also found at the *D11S1313* and *D11S901* loci which map to 11cen and proximally on 11q, respectively. In four cases, LOH at *D11S1313* or *D11S901* was a distinct event from allele loss at *NCAM* or *D11S29*, and it remains possible that a second tumour suppressor exists proximally on 11q. There are few candidate loci specific for melanoma tumorigenesis on 11q, although the multiple endocrine neoplasia (*MEN1*) locus maps to 11q13 and is a candidate owing to the neural crest origin of melanocytes. Of the markers studied, *D11S901* is probably the nearest to *MEN1*.

There is acccumulating evidence that LOH on chromosome 11q is associated with metastatic melanomas [6, 11]. Our results are consistent with this possibility: although only four of the 32 tumours studied were primary lesions, no primary tumour had lost an allele at *D11S29* and only one showed LOH at *NCAM*. Tomlinson and colleagues [5] showed a correlation between allele loss at *D11S29* and early age of presentation with melanoma, but no associations were found between the clinical data and LOH in the samples studied here.

Six of 32 (19%) tumours showed evidence of replication errors (RERs). Of these, three (9%) had RERs at two or more loci, compared with an equivalent frequency of about 10–20% in sporadic colorectal cancers (e.g [23]). Two melanomas, in particular, had evidence of microsatellite instability at five and six loci, respectively, out of eight loci studied in total. Previous reports have suggested that RERs in melanomas are confined to novel tumour alleles that differ by just one repeat unit from normal, and occur in a small proportion of microsatellite loci studied [6, 20]. The frequent RERs seen in cases 7 and 15, with differences from the normal alleles of more than one repeat unit, are unusual in melanomas (Figure 3). These patterns of microsatellite instability closely resemble those in tumours from HNPCC patients. However, no mutations or allele loss were found at the *hMSH2* and *hMLH1* loci in tumours 7 and 15. It remains possible that other mismatch repair loci (such as *hPMS1* and *hPMS2*) were mutated in these tumours, or that SSCP analysis failed to detect mutations at *hMSH2* and *hMLH1*. However, given that melanoma is not generally considered to be part of the HNPCC syndrome, the RERs observed in tumours 7 and 15 may reflect mutations at genes which do not cause HNPCC, but which can still lead to microsatellite instability.

Allele loss on chromosome 11q and microsatellite instability are both found in several tumour types. There are several examples of mutations common to melanomas and to other tumours: for example, mutation of the *p16* gene, perhaps

involved in familial cases of melanoma [24–27], occurs frequently in tumours from many different tissues of origin [28]; and allele loss on chromosomes 1p [29, 30] and 6q [4, 31] is common in melanoma and in other cancers. It appears, therefore, that the genetic pathways of melanoma tumorigenesis and those of other cancers contain areas of overlap. Chromosome 11q may contain a tumour suppressor gene with importance not only for melanoma, but also for other types of cancer.

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