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Frequency of homozygous deletion at *p16/CDKN2* in primary human tumours

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Many tumour types have been reported to have deletion of 9p21 (refs 1-6). A candidate target suppressor gene, *p16* (*p16*^{INK4a}/*MTS-1/CDKN2*), was recently identified within the commonly deleted region in tumour cell lines[7,8]. An increasing and sometimes conflicting body of data has accumulated regarding the frequency of homozygous deletion and the importance of p16 in primary tumours. We tested 545 primary tumours by microsatellite analysis with existing and newly cloned markers around the *p16* locus. We have now found that small homozygous deletions represent the predominant mechanism of inactivation at 9p21 in bladder tumours and are present in other tumour types, including breast and prostate cancer. Moreover, fine mapping of these deletions implicates a 170 kb minimal region that includes *p16* and excludes *p15*.

According to Knudson's hypothesis[9], both alleles of a suppressor gene are independently inactivated, generally by point mutation of one allele and deletion of the other wild-type allele. Point mutation of *p16* has been described in melanoma kindreds, pancreatic, and some esophageal and lung cancers[10–13]. However, there have also been negative findings reported for some of these tumours[14–17]. In common neoplasms with frequent loss of heterozygosity (LOH) at 9p21, point mutation of *p16* is rare or absent[18–20]. Although, homozygous deletions have been described in many tumour cell lines, only a minority of primary tumours with 9p21 LOH have been found to contain homozygous deletion. The paucity of point mutation and apparent low frequency of homozygous deletion in primary neoplasms with frequent LOH of 9p21 have led to suggestions that *p16* may not be the only (or primary) target of inactivation in this region.

Table 1 Allelic status of 9p21 in primary tumours

Tumour source	Total	Any Loss of 9p[d] (%)	Total[e] LOH only	Total[e] homozygous deletion (%)
Bladder	285	177 (62)	51	126 (71)
Head and Neck	65	49 (75)	33	16 (33)
Renal Cell	45	18 (40)	11	7 (39)
SCLC[a]	39	30 (77)	28	2 (7)
NSCLC[b]	39	31 (79)	26	5 (16)
Breast	20	13 (65)	11	2(15)
Prostate	15	8 (53)	5	3(40)
Endometrial	18	5 (28)[f]	5	0 (0)
Cervical	10	2 (30)	2	0 (0)
Colon	9	3 (33)	3	0 (0)
	545			

[a]Small Cell Lung Cancer; [b]Non-Small Cell Lung Cancer; [c]Most microdeletions (hemi- or homozygous losses) were documented only by testing the new markers described in this study (*D9S1747-52*). For bladder cancer, 96 of the total 126 homozygous deletions were detected only by using these markers; [d]Tumours with monosomy or loss of 9p only (selective 9q losses are excluded); [e]These may in fact be small homozygous deletions (see text); [f]Microsatellite alterations of individual loci in several RER$^+$ tumours were counted as noninformative in this study.

We previously mapped chromosome 9 deletions extensively with available markers in bladder tumours[5] and observed a homozygous deletion rate of 11% (30 of 285). In this study, we tested 5 newly isolated microsatellite markers (Fig. 1*a*) in all 285 tumours and identified 96 new homozygous deletions by apparent retention of heterozygosity in one or more closely spaced markers within a region of LOH[5]. This apparent retention of heterozygosity results from amplification of a small amount of normal cells within the tumour since the homozygously deleted region within the neoplastic cells does not provide a template for amplification. In total, 126 of 178 (71%) tumours with LOH through 9p21 contained a detectable homozygous deletion (Fig. 1*b,c* and Table 1). Only 30/126 (24%) of the deletions in bladder tumours were identified without use of these new polymorphic markers. *D9S1747* and *D9S1748*, the closest flanking markers to *p16*, showed the highest frequency of homozygous deletion and also detected 15 new hemizygous deletions. Moreover, *D9S1748* maps approximately 15 kb proximal to *p16* (Fig. 1*a*), and 25 bladder tumours contained homozygous deletions that did not extend to this marker and therefore excluded *p15*.

Retention of a polymorphic marker by Southern analysis is a more reliable method to assess homozygous deletion than using a non-polymorphic probe followed by stripping and reprobing the filter with a control probe for comparison of the relative signals. An anonymous RFLP marker, *CN2* (ref. 21), was localized proximal to *D9S1749* and near *D9S1747* (Fig. 1*a*). Using Southern analysis, 103 bladder tumours were informative for this RFLP and the results were concordant with the heterozygosity status at *D9S1747*. Retention or amplification of alleles at 9p21 was therefore excluded as a possibility for the apparent retention of heterozygosity detected by microsatellite analysis.

Fluorescent *in situ* hybridization (FISH) may be the most reliable method of detecting homozygous deletions since normal cells can be distinguished from neoplastic cells and homozygous deletion from retention of alleles. Because FISH is also the most laborious method, we only performed this analysis on nine primary tumours. FISH identified eight cases of homozygous deletion and one case of hemizygous deletion of *p16* which correlated precisely with results from the microsatellite markers for these tumours (Fig. 2) and further confirmed that apparent retention of heterozygosity represents homozygous deletion.

We investigated further the role of 9p21 deletion in other tumour types (Table 1). In a preliminary analysis of neoplasms where homozygous deletion had been previously described (lung, head and neck, renal cell), the frequency of deletions (either hemi- or homozygous) noticeably increased using the new microsatellite markers in the same cohort of tumours. Moreover, these microsatellite markers identified homozygous and hemizygous deletion for the first time in primary breast and prostate tumours. Many deletions of 9p21 were found to be under 200 kb in size in this study and would have been missed without testing markers close to *p16*. A recent FISH analysis of bladder tumours identified few homozygous deletions with *IFNa* as a probe, only 500 kb away from *p16* (ref. 22). We have found other methods such as multiplex PCR of the *p16* region to be unreliable

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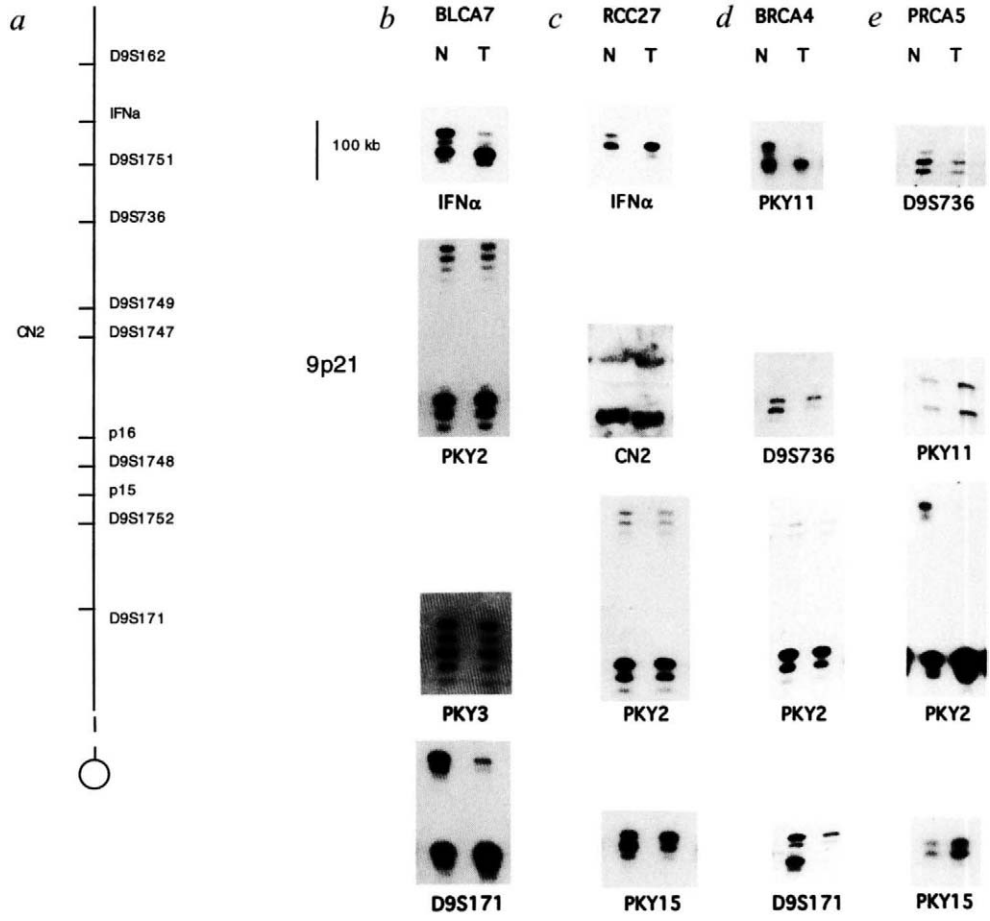

Fig. 1 *a*, Map location of 9p21 markers. The previously mapped markers *D9S162*, *IFNa*, *D9S736* and *D9S171* are indicated together with selected newly cloned polymorphic markers, *D9S1747-52*. The map location of the RFLP (CN2) is also shown. An approximate kilobase (kb) scale is indicated on the right. Five highly informative microsatellites (*D9S1747-52*) were chosen at particular intervals of the contig and used to test normal and tumour DNA pairs for heterozygosity status. *b–e*, Demonstrations of homozygous deletions by microsatellite analysis. *b*, Bladder tumour 7 showing apparent retention of heterozygosity indicating homozygous deletion at *PKY2* (*D9S1747*) and *PKY3* (*D9S1748*) flanked by LOH, indicated by loss of the upper allele, at IFNa and D9S171. *c*, Renal cell tumour 27 with homozygous deletion at *CN2* (tumour lane is overloaded) and *PKY2* (*D9S1747*) flanked by LOH, indicated by loss of the upper allele at *IFNa* and the lower allele at *PKY15* (*D9S1752*). *d*, Breast tumour 4 with homozygous deletion at *PKY2* (*D9S1747*) flanked by LOH, indicated by loss of the upper allele at *PKY11* (*D9S1751*) and the lower allele at *D9S736* and *D9S171*. *e*, Prostate tumour 5 showing LOH, indicated by loss of the upper allele at *PKY2* (*D9S1747*) with the flanking markers *D9S736*, *PKY11* (*D9S1751*) and *PKY15* (*D9S1752*) showing retention of heterozygosity.

in many tumours because of poor amplification of GC-rich areas in *p16*, duplicated sequences and variable amounts of contaminating normal tissue. Moreover, we have found Southern analysis of non-polymorphic probes to be definitive only in ideal circumstances.

We have previously observed that exon 1 of *p16* was often not included in the homozygously deleted area by Southern analysis[18]. This observation has also been made independently by others in cell lines[23] and supports our observation that many deletions may extend only into the 3' end of *p16*. One hypothesis proposes that the high frequency of homozygous deletion at this locus may be the preferred mechanism of inactivating alleles from two suppressor genes by one deletion event[20]. Although our data argues against *p15* as the second target at 9p21, we cannot exclude a second and distinct suppressor target in addition to *p16*. The non-random distribution of microdeletions may also imply the existence of an underlying structural mechanism. Indeed, germline microdeletions were observed at a locus on chromosome 5q linked to spinal muscular atrophy[24,25]. This locus shares certain structural features with 9p21 including the presence of duplicated genes and a high density of repeat sequences.

The frequency of homozygous deletion within the 170 kb minimal region at 9p21 is still likely to be an underestimate in our study of primary cancer for a number of reasons; (i) in a number of cases, patients are non-informative at critical loci such as *D9S1747* and *D9S1748*;

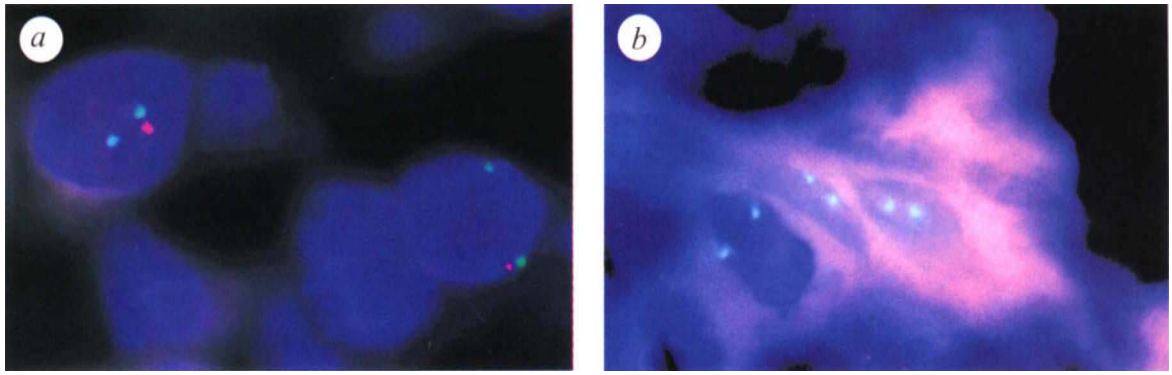

Fig. 2 FISH of interphase nuclei from bladder tumours. *a*, Hemizygous deletion of *p16* indicated by the presence of two signals for the centromere probe of chromosome 9 (green) and one signal for the 2230 P1 clone containing *p16* (red). *b*, Homozygous deletion of *p16* indicated by two signals for the centromere of chromosome 9 (green) and no detectable signal for the 2230 P1 containing *p16* (red). In both tumours LOH confined to 9p was demonstrated by microsatellite analysis. FISH was performed independently, without knowledge of the deletion status of the tumours, using a P1 clone, (2230, containing the entire genomic sequence of *p16*) simultaneously with a probe for the centromere of chromosome 9 as a control.

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(ii) since the deletions appear to be nested, microdeletions of *p16* not extending to *D9S1747* or *D9S1748* remain to be detected; (iii) the microhemizygous deletions may have a second nested deletion which cannot be detected by the spacing of the currently available markers; and (iv) in bladder and lung cancer, monosomy of chromosome 9 is much more frequent than selective deletion of the p arm. Reports of discrete q deletions imply the presence of a suppressor locus on the q arm, and thus some monosomies may target this gene and not *p16* (ref. 26).

Although deletion of both alleles was previously proposed as the predominant inactivation mechanism for *p16*, it was necessary to accurately assess the frequency and extent of homozygous deletion in primary tumours with 9p21 LOH. We have convincingly demonstrated that primary tumours harbor homozygous deletion at 9p21 and the absence or rarity of point mutation of *p16* in tumours with 9p21 LOH is therefore no longer a sufficient argument against *p16* as the critical target gene. In most of these neoplasms, deletion at 9p21 far surpasses any other established genetic event in primary tumours and agrees with earlier observations in many cell lines[7,8]. Although we cannot rule out the presence of a second suppressor locus at 9p21, *p16* appears to be at least one target of inactivation in many neoplasms. Homozygous deletion, methylation[27] and rare point mutation of *p16* may account for the other inactivation event in many tumours with loss in this region, confirming the role of this cyclin/CDK inhibitor in the progression of primary human cancers. However, all of these events are rare in some tumours with 9p21 loss (for example, SCLC) and further characterization of the timing and frequency of each of these *p16* inactivation events in different cancers remains a high priority.

Methods

Construction of a P1/cosmid contig and isolation of microsatellite markers. A contig extending from *IFNa* through *p15* was constructed by initially screening a flow sorted human chromosome 9 cosmid library and a total human P1 library with the reference markers *IFNa*, *D9S736* and *p16*. Subsequently, a complete P1 contig of the region was published[28] and 2 of these P1 clones were generously provided to close our contig. Restriction fragment fingerprinting confirmed that the contig was complete. Individual clones from the contig were screened with microsatellite repeat oligomers, CA, AG, AAN and AAAN to detect polymorphic blocks. After isolation, markers were mapped and ordered by oligo-probing of Southern blots of restriction fragment fingerprints of the contig (Fig. 1*a*).

Specimens and DNA extraction. Primary tumour specimens were obtained after surgery and frozen immediately. Peripheral blood from each patient was collected in EDTA as a normal control. Macroscopically pure tumour was used or microdissection by cryostat sectioning was performed as described to isolate neoplastic cells from fresh frozen biopsies[6]. A few samples were obtained in paraffin and isolated as described. Leukocytes were pelleted from blood samples before extraction and purification of DNA[6].

Polymerase chain reaction (PCR) amplification. DNA from tumour and venous blood was analyzed for LOH or homozygous deletion by amplification of dinucleotide repeat-containing sequences using PCR and the conditions previously described[6]. Homozygous deletions were scored when one or more closely spaced markers demonstrated apparent retention when flanked by markers demonstrating clear loss of heterozygosity. All primer sequences are available from Research Genetics (Huntsville, AL) (*D9S162, IFNa, D9S171, D9S736*) or from the Genome Database (JHU, MD) (*D9S1747-52*).

Southern analysis and DNA probes. Restriction digestion of genomic DNAs, blotting, labelling of the probe fragment, hybridization and stripping of filters were carried out as previously described[5]. A 1.6-kb genomic fragment from pCN2 was used to probe *Taq*I digests[21].

FISH procedure. Frozen tissue was sectioned onto slides and dried. Slides were dehydrated in an ethanol series, denatured in formamide, dehydrated in an ice-cold ethanol series and air dried[29]. The hybridization solution contained CoT1 DNA, SpectrumOrange *p16* probe, and SpectrumGreen *CEP9* probe. After overnight incubation at 37 °C, slides were washed and counterstained with DAPI. 250 nuclei were analyzed and images captured by a SmartCapture System (Vysis, Framingham, MA) mounted on a Zeiss Axiophot microscope.

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