Loss of heterozygosity in tumor cells requires re-evaluation: the data are biased by the size-dependent differential sensitivity of allele detection

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Abstract Normal tissue contamination of tumors may eclipse the detection of loss of heterozygosity (LOH) by microsatellite analysis and may also hamper isolation of tumor suppressor genes. To test the potential impact of this problem, we prepared artificial mixtures of mouse-human microcell hybrid lines that carried different alleles of the same chromosome 3 marker. After performing an allele titration assay, we found a consistent difference between the LOH of a high molecular weight (H) allele and the LOH of a low molecular weight (L) allele of the same CA repeat marker. It follows that normal tissue admixtures will be less of a problem when LOH affects a H allele than with a L allele. Random screening of 100 papers published between 1994 and 1999 revealed that the loss of a L allele was recorded at about half the frequency (52%) of loss of a H allele. To avoid this bias, we have developed rules for the evaluation of LOH data. We suggest that the loss of a L allele should be given more weight than the loss of a H allele in LOH studies using microsatellite markers.

Key words: Human genetics; Loss of heterozygosity; Tumor suppressor gene; Microsatellite marker; Human chromosome 3; Interstitial deletion; Renal cell carcinoma

1. Introduction

Loss of heterozygosity (LOH) is frequently used as an indicator of genetic loss associated with tumor development and CA repeat analysis using polymorphic microsatellite markers is often the method of choice for LOH detection. LOH studies performed on the same chromosomal region of the same type of tumors have often led to different results in different laboratories. Reports differ, for example, on the extent of 3p losses in renal cell carcinoma (RCC) and other solid tumors, with some papers reporting large terminal deletions and others claiming interstitial deletions [1,2]. Reports of the frequency of LOH can also differ for the same marker in the same type of tumor. For instance, D3S1289 was deleted in only 20% of RCC cases studied by Lubinski et al. [3], but in all the cases studied by Wilhelm et al. [4]. Foster et al. [5] reported deletion of marker D3S1317, the VHL gene, in only 20% of cases, whereas 84% of the cases studied by Shuin et al. [6] showed deletion of this marker.

The admixture of stroma, blood vessels, lymphocytes and other normal cells in a tumor is an unavoidable source of error in LOH studies of solid tumors. If different markers are affected to varying degrees by this admixture, spurious LOH patterns may be obtained. We were alerted to this problem in a previous study of nasopharyngeal carcinoma (NPH) [7]. Multiple interstitial deletions were found on the short arm of chromosome 3 in several tumors, chromosome 3 markers were lost with frequencies that ranged from 9 to 60% of the cases. We have referred to this effect as the 'zebra pattern'. The question arose whether this pattern is an artifact and reflects differences in the sensitivity of detection with different markers (where one marker is more affected by normal tissue contamination than another) or reflects true cases of interstitial deletions.

In the study of NPC [7], we compared the sensitivity of detection of three dinucleotide markers (D3S1217, D3S1297 and D3S1304) by PCR. These markers were selected because they showed different frequencies of LOH in the study (58% for D3S1217, 33% for D3S1297 and 9% for D3S1304). After serially diluting the human DNA with mouse DNA and amplifying the DNA using different primers, no major differences in sensitivity were detected. The aim of the present study was to investigate this problem in more detail.

2. Materials and methods

2.1. Cell lines, DNA samples and general methods

DNA was extracted from the mouse-human microcell hybrid (MCH) lines using the standard phenol extraction method and used in the allele titration assay (ATA) [8,9]. The cell lines MCH903.1 and MCH939.2 were used for most of this study. MCH903.1 is a MCH line that contains a single copy of human chromosome 3, derived from a normal human diploid cell line (HFDC), as its only human component [8,10,11]. The MCH939.2 cell line [8,10,11] originally contained a cytogenetically normal chromosome 3 (derived from a normal human diploid cell line HHW1108), but now carries a small deletion in the short arm of this chromosome (3p21.3-p22).

Paired normal and tumor RCC tissue samples were obtained immediately after resection and stored at −80°C before DNA extraction. Each tumor piece was examined histopathologically and only clear cell type tumors were used. After DNA extraction, the DNA integrity was checked by agarose gel electrophoresis.

2.2. Microsatellite analysis

PCR primers were from Nordic Primer Resource (Dept. of Clinical Genetics, Univ. Hospital, Uppsala, Sweden) and Life Technologies (Gibco BRL, UK). PCR markers used in the study are assigned (hyperlink http://gdbwww.gdb.org/) to 3p26-p25 (D3S1297, D3S1038,

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Abbreviations: ATA, allele titration assay; H allele, high molecular weight allele; L allele, low molecular weight allele; LOH, loss of heterozygosity; MCH, mouse-human microcell hybrid line; NPC, nasopharyngeal carcinoma; RCC, renal cell carcinoma

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D3S1317, D3S1304 and D3S1286), to 3p24 (D3S1620, D3S1283), to 3p22 (D3S1611, D3S1298, D3S3527), to 3p21 (D3S1568, D3S2420, D3S2409, D3S2456, D3S1767, D3S1766, D3S1289), to 3p14 (D3S1285, D3S1217), to 3p13 (D3S2454), to 3p12 (D3S2406), to 3q13 (D3S1278), to 3q21-3q25 (D3S47), to 3q27 (D3S1265) and to 3q28 (D3S1314 and SST).

PCR was performed in a 15 μl reaction volume, containing 2.5–5.0 pmol of each primer, 0.2 mM of each dNTP, 50–200 ng template DNA and 1 U *Taq* DNA polymerase. One of the paired primers (0.25 pmol) in the reaction mixture was end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The DNA samples were amplified by 30 PCR cycles comprising 1 min denaturation at 94°C, 1 min at the appropriate annealing temperature and 1 min at 72°C, in a Techne PHC-3 thermal cycler and Perkin Elmer GeneAmp PCR System 2400 (Foster City, CA, USA).

According to the size of microsatellite marker, electrophoresis was carried out in a 4.5–6.5% acrylamide/7 M urea gel at 60°C at a constant power of 60 W. After electrophoresis, the gels were exposed to X-ray film at −80°C (Fuji, Tokyo, Japan). Densitometry was then performed using a Molecular Dynamics Personal Densitometer SI (Sunnyvale, CA, USA) according to the manufacturer's protocol.

3. Results and discussion

3.1. ATA studies

Using the five available MCH lines that contained chromosome 3 from different patients [8,9], we performed an ATA using DNA samples mixed in a ratio that mimicked the mixed cell population found in tumor biopsies. DNA from two MCH lines that carry different alleles at specific loci was mixed in different proportions and then analyzed by PCR using six microsatellite markers (D3S1217, D3S1283, D3S1289, D3S1297, D3S1304 and D3S1317) distributed along the short arm of chromosome 3. Some of these markers were also used in our previous study [7].

All markers gave very similar titration curves in the ATAs (e.g. D3S1304 and D3S1217, Fig. 1). This is consistent with our conclusion from our previous study [7] that different markers are detected with similar sensitivities. However, analysis of the data indicated an unexpected systematic difference between the titration curves of the high and low molecular weight (H and L) alleles of the same marker (Fig. 1). A decrease of the H allele signal could be detected if the frequency of the 'contaminating normal cells' was less than 80% (60% or more of the L allele). In contrast, loss of the L allele could only be detected if the frequency of the 'contaminants' was less than 60% (70% or more of the H allele).

All the assayed markers showed a similar allele detection asymmetry, although to various degrees. The reasons for this asymmetry may be related to the non-linearity of the radioautographic signal on X-ray films (as described in the Amersham Catalogue), although a number of other factors may contribute to this phenomenon, for example, the stronger signal of the L allele compared with the H allele. Using DNA isolated from three healthy individuals, we measured the ratio between the L and H alleles of eight random CA repeat markers (D3S1265, D3S47, SST, D3S1278, D3S1314, D3S1297, D3S1611 and D3S1620). In all cases, the ratio of these alleles was higher than 1, with an overall average ratio of 1.73. This is surprising: usually, sequences amplified with the same PCR primers producing products with minimal differences in length (e.g. 250 and 254 bp) would not be expected to exhibit different PCR amplification rates. Conceivably, *Taq* polymerase may not discriminate between amplification products of 250 and 254 bp, but may discriminate between $(CA)_n$ and $(CA)_{n+2}$ repeats. This may be related to the 'slippage' of *Taq* polymerase (as well as other DNA polymerases) when it amplifies stretches of simple nucleotide repeats. It has also been noted that simple repeats can cause replication blockage in vivo [12]. Stronger signals for the L allele have also been mentioned earlier [13,14].

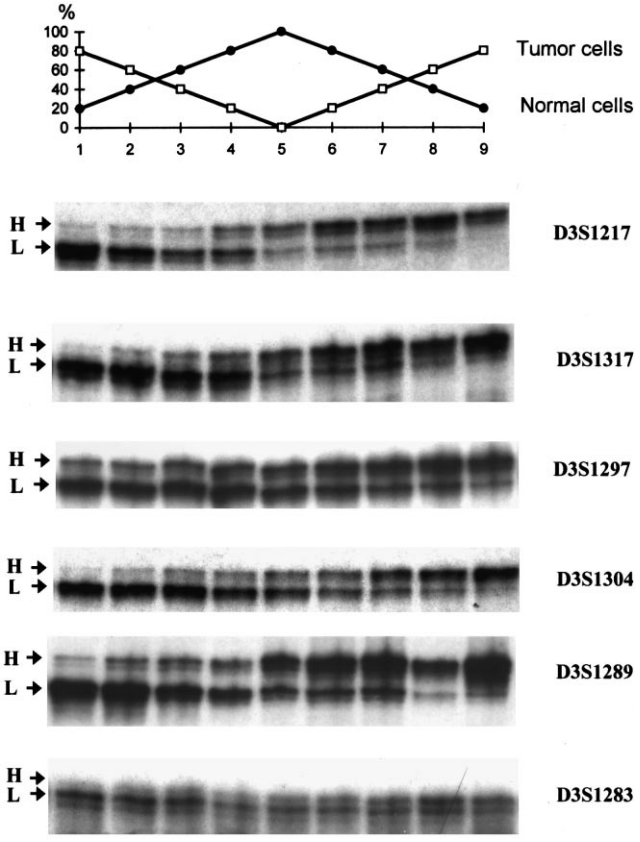

Fig. 1. Microsatellite analysis of six CA-polymorphic markers. DNAs for ATA were obtained from MCH lines [8–11]. Lanes 1–9 contain from 10 to 90% of DNA with H allele, respectively. The upper part of the figure shows correlation between the content of H and L alleles and 'tumor/normal DNA'. The loss for allele H could be unambiguously detected up to 60% of normal cell admixture, but not at 80% or more. In contrast, loss of the L allele could only be detected if the normal cell admixture was less than 40%. It could not be observed at 60% or more. This bias implies that normal cell admixture obscures the detectability of LOH from tumor cells to a different extent, depending on whether the loss affects the L or the H allele of the same locus.

The existence of shadow bands (generated when *Taq* polymerase slips over short tandem repeats thus producing shorter products) may be another factor affecting the asymmetry of allele detection. Shadow bands from a H allele may increase the strength of a L allele. We measured the signal intensity of H and L bands of all the markers tested in this study, using a Molecular Dynamics Personal Densitometer SI. For the dilutions in which the proportion of H allele was 10–50%, the ratio of signal intensity (HR = H/L) between H and L was calculated for five points (1 = 10% of H and 90% of L allele, 2 = 20% of H and 80% of L, etc.). For the DNA ratios where the L allele represented 10–50% of the mixed DNA sample, the ratio of signal intensity between the L allele and the H allele (LR = L/H) was given a similar point score (1 = 10% of L allele, etc.). The ratio between LR and HR was calculated for each of the five points, for all markers.

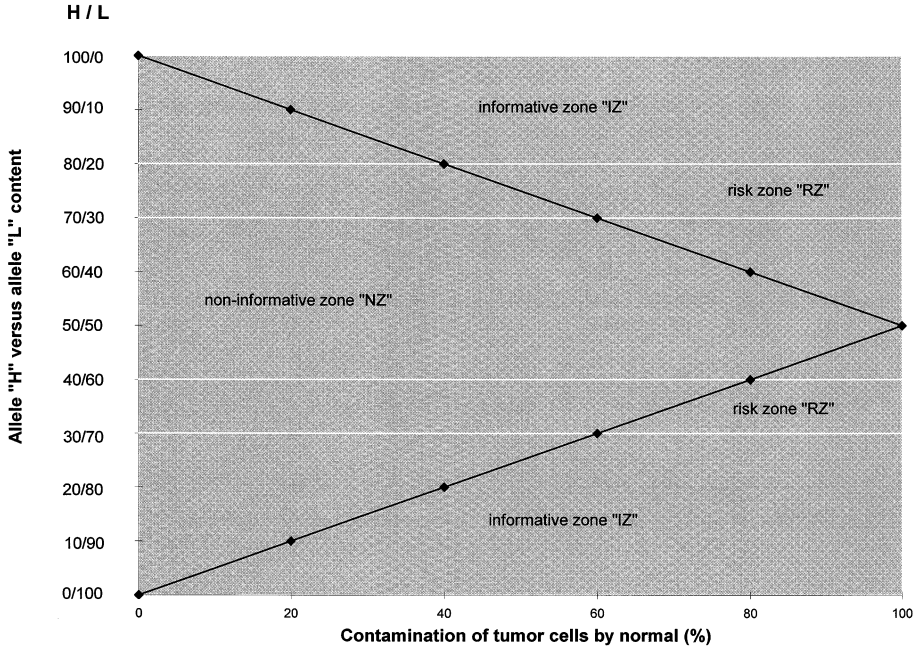

Fig. 2. Summary data from the dilution experiments. They are shown together with theoretical curves representing the deletion of one allele at different proportions of H and L alleles. For example, 10% of one allele in our dilution experiments corresponds to 20% of contamination of tumor with normal cell DNA. The figure shows the differences between H and L allele detection at the same level of normal DNA contamination and indicates the informative zone where LOH is clearly detectable, a non-informative zone where it cannot be detected, and a risk zone where its detectability is ambiguous.

The ratio of LR to HR was maximal when the mixed DNA sample contained 20–30% of either of the two alleles (Fig. 1, lanes 2 and 3 compared with lanes 7 and 8). This range defined the zone of highest sensitivity for the detection of H allele loss compared with L allele loss, suggesting that in samples that contain 40–60% normal cells, H allele loss may be detected, but L allele loss may be missed. Normal cell admixtures, therefore, may obscure the detection of LOH in tumors to a varying extent, depending on whether the loss affects the L or the H allele of the same locus. In Fig. 2, the data from the mixed DNA samples are presented with the theoretical curves for allele deletion in the presence of varying proportions of H and L alleles. For example, 10% of one allele in the mixed DNA sample corresponded to 20% normal cell DNA contamination of the tumor sample, 20% of either L or H alleles in the DNA mixture corresponded to 40% normal cell contamination, etc.

Fig. 2 shows the differences between detection of the H and L alleles at the same normal DNA contamination levels. Loss of the H allele could be unambiguously detected in the mixed DNA samples containing up to 60% of normal cell DNA, but H allele loss could not be detected when the normal cell DNA represented 80% or more of the sample. In contrast, loss of the L allele could only be detected when the normal cell admixture represented 40% or less of the sample. L allele loss could not be observed when the normal cell admixture formed 60% or more of the DNA sample.

Fig. 2 indicates a series of zones. The first zone represents the informative region of the curve, where LOH can be clearly detected. A non-informative zone, where LOH cannot be detected, and a risk zone, where detection of LOH is ambiguous, are also apparent. In LOH analysis of real tumor samples, it is not known whether the H or the L allele of a given marker is deleted. As tumors may carry 20–60% admixed normal cells, many cases will be in the risk zone. The upper limit of the risk zone for the L allele is usually 40%, but in some cases may be as low as 20%. In these cases, the risk zone may be significantly greater. This implies that unambiguous LOH data can only be obtained from samples containing less than 20% contaminating material and the probability of detecting a LOH in the presence of contaminating normal cells is higher with regard to the H allele compared with the L allele.

3.2. *Difference in detection of allele losses and three rules for LOH analysis*

If the bias described in this study has influenced the LOH studies of solid tumors, we would expect that losses of the H allele were more frequently detected than losses of the L allele. We have checked 100 randomly selected papers published between 1994 and 1999 that included LOH studies. Table 1 shows the reported loss of the H allele versus the L allele. Altogether, the studies report 530 cases with deletion of the H allele and 275 cases with L allele deletions. Statistical analysis of these results based on z approximation ($z = 8.95$) of a binominal test (two-tailed $P \ll 0.00001$) showed that the observed difference in detection of allele loss is highly significant. For comparison, at $z = 4$, P is less than 0.00003. These results suggest that about 50% of the L allele deletions in tumor samples may go undetected.

Our preliminary data suggest that the conclusions drawn for dinucleotide markers also apply to trinucleotide and tetranucleotide markers. We analyzed our recently published data of deletion mapping of 3p in different epithelial tumors [115] using trinucleotide and tetranucleotide markers for a total of seven loci. We found that, in epithelial tumors, L allele deletions were also detected less frequently than H allele deletions (99 of L allele loss versus 142 of H allele, $z = 2.7$, $P = 0.0035$). Although this study of epithelial tumors was not

Table 1
Frequencies of losses of H versus L alleles

Study no.	Number of H allele losses	Number of L allele losses	Reference
1	4	2	[15]
2	1	3	[16]
3	8	5	[17]
4	4	1	[18]
5	3	2	[19]
6	6	6	[20]
7	6	3	[21]
8	6	3	[22]
9	5	4	[23]
10	6	2	[24]
11	2	2	[25]
12	5	3	[26]
13	5	3	[27]
14	3	2	[28]
15	4	1	[29]
16	6	2	[30]
17	5	1	[31]
18	15	7	[32]
19	2	2	[33]
20	3	1	[34]
21	1	3	[35]
22	11	1	[36]
23	5	5	[37]
24	4	6	[38]
25	5	4	[39]
26	11	8	[40]
27	3	0	[41]
28	6	5	[42]
29	6	2	[43]
30	1	3	[44]
31	5	2	[45]
32	5	3	[46]
33	11	7	[47]
34	16	8	[48]
35	3	1	[49]
36	5	1	[50]
37	4	1	[51]
38	5	1	[52]
39	4	0	[53]
40	3	2	[54]
41	6	4	[55]
42	4	3	[56]
43	4	2	[57]
44	3	3	[58]
45	3	1	[59]
46	6	3	[60]
47	6	4	[61]
48	7	3	[62]
49	8	6	[63]
50	10	5	[64]
51	2	5	[65]
52	4	3	[66]
53	7	3	[67]
54	2	3	[68]
55	11	4	[69]
56	4	2	[70]
57	4	2	[71]
58	4	0	[72]
59	1	5	[73]
60	4	1	[74]
61	7	1	[75]
62	3	2	[76]
63	3	2	[77]
64	4	2	[78]
65	3	1	[79]
66	4	4	[80]
67	13	5	[81]
68	4	1	[82]
69	5	1	[83]
70	2	3	[84]
71	13	2	[85]
72	8	2	[86]
73	12	7	[87]
74	2	2	[88]
75	5	5	[89]
76	3	1	[90]
77	2	2	[91]
78	10	6	[92]
79	4	2	[93]
80	3	2	[94]
81	4	3	[95]
82	6	1	[96]
83	12	5	[97]
84	8	0	[98]
85	10	2	[99]
86	2	0	[100]
87	11	7	[101]
88	6	4	[102]
89	3	1	[103]
90	4	1	[104]
91	9	6	[105]
92	2	1	[106]
93	3	0	[107]
94	8	1	[108]
95	1	2	[109]
96	5	5	[110]
97	4	1	[111]
98	2	0	[112]
99	4	2	[113]
100	3	0	[114]
Total	530	275	
	275:530 = 0.52		

very extensive, we think that the difference in H and L allele detection we observed is important because the tumor samples were purified by microdissection and the L to H allele ratio for the CA repeat markers was 58:72 ($z = 1.14$, $P = 0.1217$).

On the basis of this study, we suggest to follow three rules for LOH analysis:

1. Comparative genome hybridization [116] or other independent methods (quantitative PCR, Southern hybridization) must be used in parallel with microsatellite analysis to exclude false 'interstitial' deletions.
2. The number of deleted H and L alleles should be counted for each paired sample of normal and tumor tissue. These numbers should be about the same.
3. Most importantly, the rule for the L allele must be taken into consideration during LOH analysis. This means that the retention of markers should be evaluated with caution, particularly if they are associated with interstitial deletions (zebra pattern). Only retained markers located in the neighborhood of the LOH involving a L allele should be evaluated as significant. Otherwise, if no independent supportive data are available, the retained marker should be considered non-informative.

As an example of how these rules can influence the results of LOH experiments, data from the RCC analysis presented in Fig. 3A have been re-interpreted and are shown in Fig. 3B. All tumor cases (T1–T4) showed deleted, non-informative and retained alleles (Fig. 3A,C). We concluded that T1, T2 and T3 had a single interstitial deletion in each sample and T4 contained two interstitial deletions (Fig. 3A). However, when we

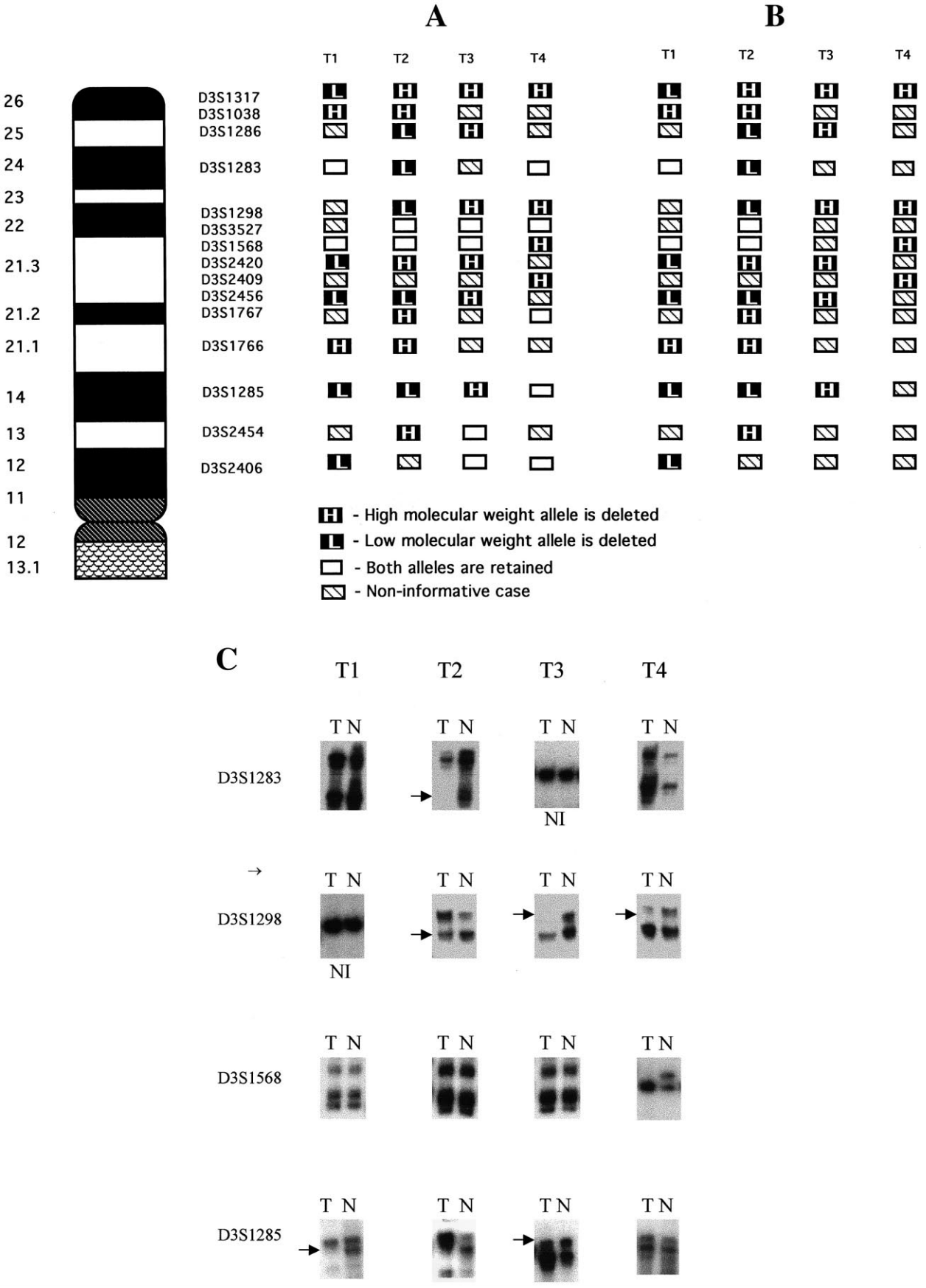

Fig. 3. Microsatellite analysis of RCC biopsies. A: Results of LOH analysis. B: Evaluation of LOH data using the ATA rules. C: Representative examples of autoradiograms demonstrating retention and LOH in RCC samples. A deleted allele is designated by an arrow; NI, non-informative.

calculated the number of H and L allele deletions, we found that for T1, the ratio was 2:5, for T2 6:5 and for T3 and T4 6:0 and 5:0, respectively. The L allele deletion (D3S2420) in T1 is close to the retained allele D3S1568 and the L allele deletion (D3S1298) in T2 is close to the retained allele D3S3527, suggesting that the interstitial deletions in these cases are genuine. At the same time, L allele deletions were not detected in T3 and T4. This suggests that these samples might have terminal deletions (Fig. 3B). To demonstrate the presence of the interstitial deletions in T3 and T4, other methods are required, such as comparative genome hybridization or quantitative PCR or ATA analysis using more microsatellite markers.

Although the rules suggested for LOH analysis in this study decrease the informativeness of microsatellite analysis, they sharply increase the confidence in informative results and help to re-evaluate the conflicting data that have been generated from a number of laboratories.

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