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Lead article

Molecular cytogenetic characterization of desmoid tumors

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Abstract Desmoid tumors are benign neoplasms of the fibromatosis group. Data on their acquired chromosomal changes are sparse and, therefore, we wanted to ascertain what genomic losses and gains these tumors may have incurred. DNA was extracted from a total of 26 formalin-fixed, paraffin-embedded desmoid tumors followed by comparative genomic hybridization (CGH) and interphase fluorescence in situ hybridization (I-FISH) analyses. Ten of 12 informative tumors were normal by CGH; the two abnormal ones had loss of chromosome 6 and loss of 6q and gain of chromosome 20, respectively. I-FISH analyses with an α-satellite probe specific for chromosome 8 of 26 desmoids, including one tumor that by karyotyping had +i(8)(q10), showed no evident abnormalities. An explanation for the relatively high frequency of genomically normal tumors by CGH seen in this study may be sought in the fact that as many as 10 of the 12 informative tumors were abdominal desmoids, a subset of tumors also previously found to exhibit genomic changes only rarely. It is therefore possible that abdominal desmoids might be non-neoplastic tumors or neoplastic tumors with genetic changes too small to be discovered by CGH, whereas desmoid tumors from other locations exhibit detectable genomic changes at a significantly higher frequency.

1. Introduction

Desmoids are deep-seated, aggressive tumors belonging to the fibromatosis group. They presumably arise from fascial or musculoaponeurotic structures and are subclassified as extra-abdominal, abdominal, and intra-abdominal [1]. Microscopically, the lesion is poorly circumscribed, with cells and collagen fibers arranged in sweeping bundles infiltrating the surrounding tissue, commonly striated muscles. Wide local excision is the treatment of choice. The incidence of desmoid tumors is two to four new cases per million per year, representing ~0.03% of all neoplasms. They occur at all ages, but mostly in young, parous women. Desmoids are unusually common in patients with adenomatous polyposis of the colon and rectum [2].

The genomic profile of desmoid tumors is not well known. To our knowledge, only 121 cases have been karyotyped [3], 50 of them in one large series [4], whereas 28 tumors have been analyzed by comparative genomic hybridization (CGH) [5]. Nearly 50% of the karyotyped desmoids have shown clonal chromosomal abnormalities [5], with +8 as the most frequent aberration [3], whereas gains of chromosome 20 and chromosome band 1q21 have been reported as the most frequent aberrations found by CGH.

We wanted to expand the existing molecular cytogenetic knowledge on desmoid tumors. Because desmoid tumors are so infrequent, the analyses almost exclusively had to be performed on archival formalin-fixed and paraffin-embedded (FFPE) tumor material. CGH was used as the main technique because karyotyping is not possible on such nonviable cells. In addition, we performed interphase fluorescence in situ hybridization (I-FISH) with a chromosome 8–specific centromeric probe to compare findings in our series with the main results obtained in previous studies of desmoid tumors.

2. Materials and methods

2.1. Samples and cytogenetic analysis

We obtained 26 FFPE desmoid tumors from the Department of Pathology at the Norwegian Radium Hospital. The

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tumors had been diagnosed at that department during the last 20 years, sufficient archival material was available from them, and the series therefore may be viewed as representative. The histologic diagnoses were reviewed and confirmed by an experienced pathologist (B.B.), who also confirmed that there was a predominance of tumor cells in each section.

One desmoid tumor was surgically removed at the Norwegian Radium Hospital while the studies on archival material were in progress, and a sample from it was processed for cytogenetic analysis. Fresh material was manually minced and treated with collagenase, hyaluronidase, and neuraminidase until a suitable suspension of cells and cell clumps was obtained. After 3 days of culturing in a medium consisting of RPMI-1640, 13% fetal calf serum, and antibiotics, colchicine was added for the last 4 hours of culturing and the short-term culture was harvested according to standard protocols [6]. The chromosomes in the dividing cells were then G-banded and a karyotype was established in accordance with International System for Human Cytogenetic Nomenclature (ISCN 1995) recommendations [7].

2.2. CGH analysis

2.2.1. DNA extraction

DNA was isolated as described previously by Kraggerud et al. [8]. Briefly, the tissue was deparaffinized in xylene, washed in alcohol, and digested in lysis buffer, proteinase K, and RNase, followed by phenol-chloroform extraction and ethanol precipitation. Several modifications of the protocol were tried to optimize the yield of high–molecular weight DNA (HMW-DNA), largely as described by Isola et al. [9]. For three of the samples, we tried prolonged proteinase K digestion (3 days instead of 1) and extraction from sections of different thickness.

2.2.2. Slide preparation

Metaphase slides were prepared as described by Karhu et al. [10] but with small modifications. Briefly, dropping of the slides was performed in a moist milieu with varying temperature, on moist or dry slides (Menzel Glaeser Superfrost Color slides, Braunschweig, Germany) with or without postfixation. The slides were evaluated according to the following criteria: adequate chromosomal length, black color, minimal overlapping, minimal cytoplasm, and adequate number of mitoses. Slides were stored at room temperature 1–4 weeks before use.

2.2.3. CGH

CGH, first described by Kallionemi et al. [11,12], was performed on 19 of the 26 tumors according to Kraggerud et al. [8] with but minor modifications. Briefly, test and reference DNA were differentially labeled with fluorochrome-conjugated nucleotides (FITC-12-dCTP and –dUTP for tumor DNA, and TexasRed-6-dCTP and -dUTP for reference DNA). Equal amounts (800 ng each) of tumor and reference DNA, denatured at 70°C for 5 minutes, together with 33 μg of human cot-DNA, were hybridized onto normal metaphase spreads denatured for 3 minutes at 70°–73°C and incubated at 37°C for 2–3 days. After washing the slides were counterstained with an antifade solution consisting of DAPI (4′,6-diamino-2-phenylindole) and Vectashields H-1200 (Vectashield; Vector Laboratories, Burlingame, CA), and examined with a fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany). Single-color images (FITC, TexasRed, and DAPI) of metaphase spreads were sequentially photographed with a Cohu 4900 CCD (12-bit gray scale) camera, using Cytovision hardware and software. Chromosomes were karyotyped on the basis of their inverted DAPI banding. Fluorescence ratio profiles (green to red) were calculated for individual chromosomes, data from 11 to 20 representative copies of each chromosome were combined, and average ratio profiles with 95% confidence intervals were calculated for each tumor. The centromeric and pericentromeric heterochromatic regions were not evaluated.

All tumors except for one were analyzed at least twice, and the profiles were evaluated independently by two researchers (P.B. and F.M.). If doubts persisted, the tumor was analyzed a third time. Poor hybridizations, mostly resulting from either suboptimal slide or DNA quality, were not accepted. One knows from karyotyping studies that desmoid tumors are mostly diploid, and we therefore chose thresholds corresponding to loss or gain of one chromosome homologue in 50% of the cells analyzed (i.e., 0.75 and 1.25, respectively).

To reduce the problem with potentially false positive areas [11–13], we used a modified CGH protocol with a mixture of fluorochrome-dUTP and -dCTP during nick translation. We also used a negative control in all experiments, and all tumor changes were evaluated against this control. Finally, the lovo cell line was used as a positive control in each experiment.

The gains and losses were described in accordance with the ISCN 1995 [7].

2.3. I-FISH

2.3.1. Samples and nucleus extraction

I-FISH was performed on a total of 26 desmoids, including the 12 from the present Norwegian series in which informative CGH results were obtained (see below) and 14 desmoids (Finnish series) with known CGH profiles [5]. The latter 14 were selected from a series of 28 desmoids to include tumors showing no aberrations by CGH ($n = 4$) as well as tumors showing aberrations ($n = 10$). The most frequent aberrations found in these 10 tumors were gains of 1q and chromosome 20 and loss of chromosome 6 [5].

The nucleus extraction method used for the two series was nearly identical, the main differences being that for tumors of the Finnish series, a shaking water bath was not used and the nuclei were spread manually. The procedures

were modified from Hedley and Liehr [14,15]. Briefly, two to four 50-μm tissue sections were deparaffinized by two changes of xylene and washed twice in absolute alcohol before rehydration. The preparations were then washed in phosphate-buffered saline (PBS) twice, prewarmed protease (Sigma Protease XXIV, 0.5 mg/mL; Sigma, St. Louis, MO) in PBS was added, and the preparations were incubated in a shaking water bath (1 hour, 37°C). The resulting cellular suspensions were then pipetted vigorously to release nuclei, filtrated through a 60-μm nylon mesh, followed by centrifugation and washing in PBS twice. Finally, the nuclear suspension was spread on poly-L-lysine–coated slides using a Cytospin3 centrifuge (Thermo Shandon, Pittsburgh, PA). The slides were left at room temperature for one night before freezing at −70°C or −20°C.

2.3.2. Pretreatment of slides

The slides were kept at room temperature from the day before the start of hybridization and pretreated as described by Knuutila et al. [16] but with minor modifications. Briefly, three changes of xylene were used to remove remaining paraffin, and then two changes of absolute alcohol were made to remove the xylene. The preparations were subsequently boiled for 15 minutes in a microwave oven and then cooled for 20 minutes at room temperature; both procedures took place with the slides immersed in sodium citrate. Finally, the slides were washed three times in PBS and dehydrated in alcohol.

2.3.3. Hybridization and analysis

A directly FITC-labeled α-satellite DNA probe for chromosome 8 and appropriate hybridization and counterstaining solutions were supplied by Cytocell (Banbury, UK). We used a protocol modified from Persons et al. [17]: briefly, the dehydrated slides were denatured in 70% formamide at 73°C for 5 minutes, dehydrated again, and dried. The α-satellite probe for chromosome 8 was directly FITC labeled and was denatured at 73°C for 5 minutes before application onto the slide. After 3 days of incubation at 37°C, the slides were washed three times: 10 minutes in 50% formamide, 10 minutes in 2× standard saline citrate (SSC), and 5 minutes in 2× SSC/0.1% NP-40. Air-drying then was carried out before application of 10 μL DAPI in antifade solution and sealing with a coverslip. Two-hundred successive whole and single nuclei were examined with a Zeiss fluorescence microscope, and the images were captured by a Cohu camera.

The specificity of the probe was validated with FISH experiments on slides with metaphases and interphase nuclei from a karyotypically normal individual.

3. Results

3.1. DNA extraction

Prolonged proteinase K digestion did not give any increase in HMW-DNA yield. The use of many thin sections

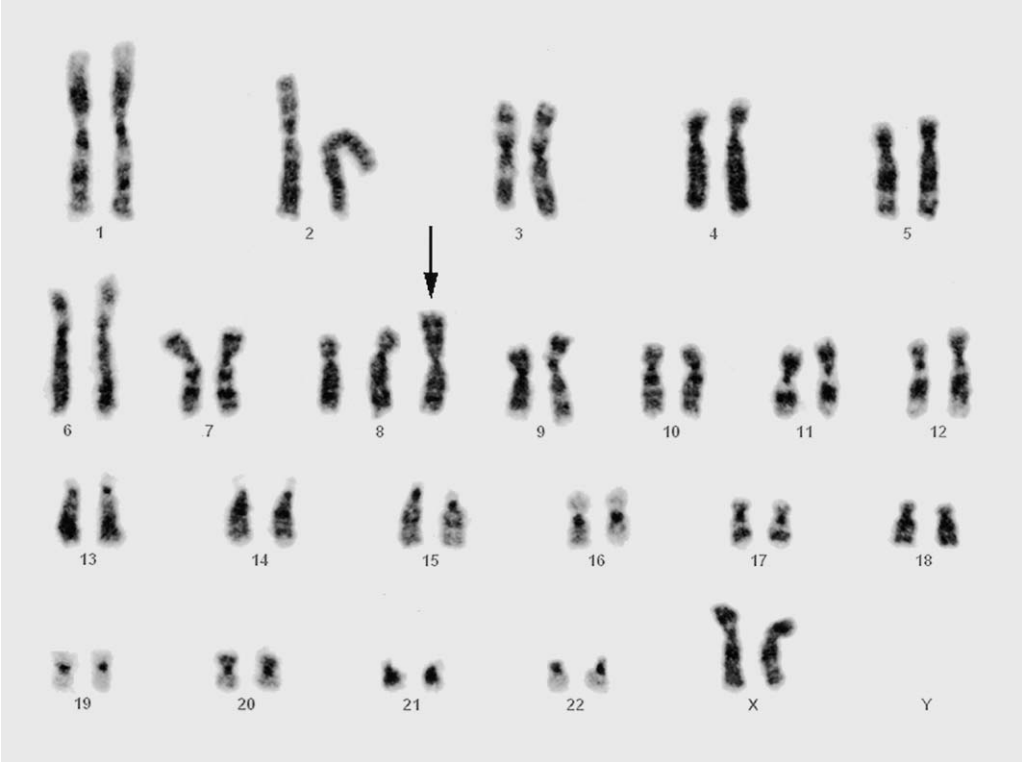

Fig. 1. Representative karyotype from case 7: 47,XX,+i(8)(q10).

(20 5-μm sections) as compared with few thick sections (five 20-μm sections) slightly increased the yield of HMW-DNA (data not shown). From 7 of the 26 tumors, no HMW-DNA at all could be obtained, and these tumors were excluded from the study. The remaining 19 tumors gave DNA concentrations of 55–172 μg/mL, all partially degraded but with some HMW-DNA as well, and were thus submitted to CGH analysis.

3.2. Karyotyping

The following karyotype was found in the one desmoid subjected to culturing and cytogenetic analysis: 47,XX,+i(8)(q10)[3]/46,XX[11] (Fig. 1).

3.3. CGH

Of the 19 tumors from which at least some HMW-DNA was available, 12 gave satisfactory hybridization by CGH, and the findings are shown in Table 1. No gains or losses of genomic material were seen in 10 of the tumors, whereas the remaining two (cases 9 and 10, Table 1) showed loss of the entire chromosome 6 and loss of chromosome arm 6q and gain of chromosome 20, respectively (Fig. 2).

3.4. I-FISH

The I-FISH data are summarized in Table 2. Of 200 counted nuclei, 167–194 had two signals, 2–20 nuclei had three signals, and the remaining mostly showed one signal for the centromere of chromosome 8. The control slide had 196 nuclei with two signals and four nuclei with one signal. There were no differences between the Finnish and the Norwegian samples. Fig. 3 visualizes interphase nuclei with one, two, and three signals for the centromeric chromosome 8 probe.

4. Discussion

Because of several potential methodological pitfalls, mostly due to suboptimal DNA quality in cells obtained from FFPE material, we tried several modifications of both the DNA extraction and CGH protocols. In our hands, prolonged proteinase K digestion did not affect the HMW-DNA yield, but the use of thin sections instead of thick sections gave some more HMW-DNA, as reported previously [9]. The differences observed might be explained by different handling of the samples during the fixation process as well as variability among tissue types. Regarding the fixation process, it has been proposed that prolonged time in formalin adversely affects the yield of HMW-DNA [9] and that buffered formalin causes less DNA damage than unbuffered formalin [18]. The samples of the Norwegian series were routinely treated with buffered formalin, but we have no knowledge about the time individual samples spent in formalin. It was therefore not possible to assess more precisely the influence of

Table 1
Clinical data and CGH findings on 26 desmoid tumors

Case no./sex/patient age (yr)	Tumor site/size (cm)	CGH findings
1/F/44	Abdominal/7	Normal
2/F/27	Abdominal/4	Normal
3/F/62	Abdominal/2	Normal
4/F/29	Abdominal/5	Normal
5/F/25	Abdominal	Normal
6/F/62	Abdominal/4	Normal
7/F/31	Abdominal	Normal
8/M/66	Abdominal/10	Normal
9/F/30	Abdominal/6	rev ish dim(6)
10/M/73	Abdominal/3	rev ish enh(20),dim(6q)
11/F/77	Shoulder/5	Normal
12/F	Not known	Normal
XP961006[a]	Abdominal	Normal
K935587[a]	Abdominal	Normal
833–80[a]	Abdominal	Normal
XP952257[a]	Abdominal	rev ish enh(9p12~p13,20), dim(6cen~q24)
XP952832[a]	Shoulder	Normal
XP923064[a]	Groin	rev ish enh(1q21,9p12)
XP916509[a]	Thigh	rev ish enh(1q21~q23,20)
XP915156[a]	Mesenterium	rev ish enh(1q21,20),dim(6)
XP943355[a]	Shoulder	rev ish enh(1q21~q23,20)
XP942732[a]	Thoracic wall	rev ish enh(1q21~q25,9p13~q21,20),dim(5q21~q22)
XP920371[a]	Mesenterium	rev ish enh(1q21~q23,20), dim(5q15~q23)
XP950647[a]	Trunk	rev ish enh(1q21~q22,9p12~q21.2,20)
XP903953[a]	Buttock	rev ish enh(1q21~q23,7p11.2~q11.2,9p12~p13,20),dim(4q,5q21~q22,6cen~q24)
XP942374[a]	Upper arm	rev ish enh(1q21,9p12~q12.2,20),dim(13q21~qter)

Abbreviations: CGH, comparative genomic hybridization; F, female; M, male.
[a] Reported by Larramendy et al. [5].

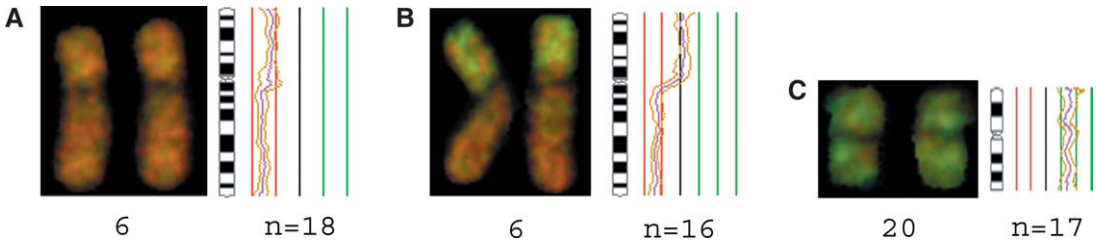

Fig. 2. Representative pairs of chromosomes and corresponding CGH profiles for chromosome 6 from case 9 (*A*), and chromosomes 6 (*B*), and 20 (*C*), from case 10. The black line represents no net gain or loss of tumor material, whereas the lines to the left (red) and to the right (green) represent 0.25 increments of losses and gains, respectively. The pink line represents the average ratio along the chromosome, the brown lines correspond to the confidence interval for the mean, and *n* is the number of chromosomes used to calculate the mean.

these parameters, beyond the fact that they might explain why we did not succeed in extracting DNA and achieve informative hybridizations from all tumors. In addition, desmoids are not particularly cell-rich tumors, something that might account for a low DNA yield.

A difference in the amount of background and the number of damaged nuclei by I-FISH was seen between the Norwegian and Finnish series. The former had less such problems, which may be explained by the fact that the Norwegian samples were spread by cytocentrifugation, whereas the Finnish samples were spread and treated more intensively manually; the latter procedure probably represents a harsher treatment of the cells. This did not represent any problem for the analysis, however, because cut nuclei were excluded.

Earlier, desmoid tumors were not considered to be true neoplasms. For example, MacKenzie [19] characterized the disease as a fibroblastic proliferation showing none of the features of an inflammatory response and no features of unequivocal neoplasia. Karlsson et al. [20] described the first abnormal karyotype in an extraabdominal desmoid tumor, and later karyotypic and clonality studies have provided evidence of the neoplastic nature of these lesions [21–26]. As a result, desmoids are now defined as benign neoplastic tumors, albeit with a potential for infiltrative growth and local recurrence causing both considerable morbidity and mortality [2,27]. The identification of genetic alterations with a potential pathogenetic impact therefore seemed to be of considerable interest.

Our present series includes 10 tumors from the abdominal wall, 1 from the scapular region, and 1 whose site of origin was unknown. In an earlier study by Larramendy et al. [5], only one of eight tumors of the abdominal wall had recognizable gains or losses of genomic material by CGH, whereas 11 of 18 desmoids from other sites had aberrations. The observed incidence of abnormalities in this series thus largely corresponds to that found by Larramendy et al. [5], in as much as only 2 of 10 abdominal tumors had aberrations, with the 2 tumors from other or unknown sites having normal CGH profiles. It seems, therefore, that a systematic, albeit quantitative pathogenetic difference may exist between extraabdominal desmoids on the one hand and abdominal desmoids on the other, with the former group of tumors more often arising as a result of recognizable genomic alterations, whereas the latter mostly are karyotypically normal and may represent pseudoneoplasms. In such a scheme, one could think that the two aberrant tumors in this series (cases 9 and 10) with an abdominal location have the same underlying pathogenetic alterations as extraabdominal desmoids. On the other hand, if abdominal desmoids are also genuine neoplasms caused by the acquisition of genomic aberrations, then these genetic changes are so small that CGH is unable to detect them. If this scenario corresponds better with reality, more detailed molecular genetic methods are necessary to find the aberrations, with one possibility being array CGH [28].

We did not observe any copy number changes at 1q21~q22 in any of the tumors examined, in contrast to

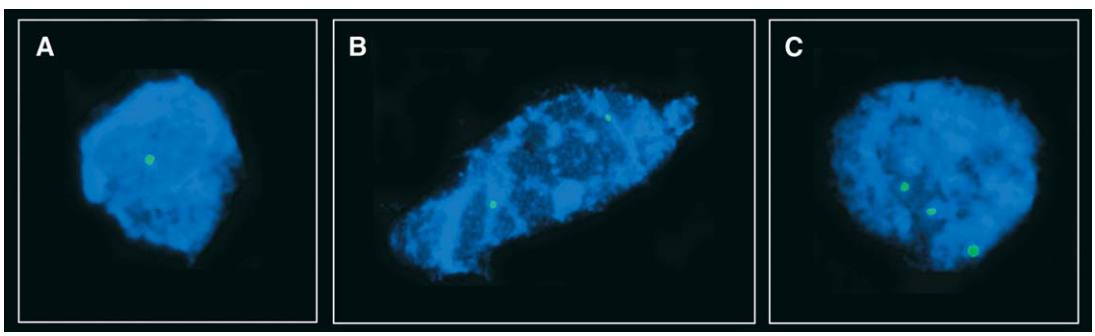

Fig. 3. (*A–C*) Interphase nuclei from case XP903953 visualizing 1, 2, and 3 signals from the centromeric chromosome 8 probe.

Table 2
Chromosome 8 I-FISH data on 26 desmoid tumors

	Signals		
Case no.	2	3	<2 or >3
1	181	14	5
2	181	13	6
3	173	15	12
4	194	2	4
5	182	7	11
6	184	11	5
7	186	6	8
8	185	5	10
9	188	5	7
10	170	17	13
11	187	4	9
12	192	4	4
XP961006	167	18	15
K935587	183	14	3
833-80	189	8	3
XP952257	186	9	5
XP952832	168	20	12
XP923064	190	5	5
XP916509	175	13	12
XP915156	186	8	6
XP943355	183	13	4
XP942732	180	13	7
XP920371	184	6	10
XP950647	178	11	11
XP903953	186	9	5
XP942374	183	9	8

Abbreviation: I-FISH, interphase fluorescence in situ hybridization.

what was seen in the Finnish series [5], again especially among the extraabdominal tumors. Since we analyzed mostly abdominal desmoids, the absence of this change in the present series is again consistent with the hypothesis that different patogenetic mechanisms may be operative in desmoids of abdominal and extraabdominal types.

Another intriguing apparent discrepancy in genetic studies of desmoids is the involvement of chromosome 8. A relatively high frequency of +8 is seen in karyotypic studies in which this gain has also been associated with a tendency for recurrence [29], whereas no involvement of this chromosome is seen by CGH [5]. Tumor 7 from the present series (Table 1) had a +i(8q) as a clonal change by karyotyping (i.e., gain of two copies of the long arm of chromosome 8) but it nevertheless had a normal CGH profile, and in the I-FISH study, 186 of 200 nuclei with two signals for the chromosome 8–specific probe were seen, whereas six had three signals. The criterion we used for accepting trisomy 8 in a sample was more than 10% of the nuclei showing three separate hybridization signals [26]. The percentage of nuclei with trisomy 8 by I-FISH in the individual samples examined by us ranged from 1 to 10%, with the average being 5%. Although none of the 26 tumors on which we performed I-FISH analysis exceeded this limit, and consequently were considered to be disomic for chromosome 8, some of the samples had values approaching 10%, and we cannot exclude the possibility that minor clones with +8 existed in these tumors. All these findings are consistent with the explanation that in most or maybe even all desmoid tumors with one extra copy of chromosome 8 by karyotyping, the extra chromosome is present only in a small clone of abnormal cells. Indeed, the +i(8q) of case 7 was seen in only 3 of the 14 metaphases examined.

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