Short Communication

Hibernomas are Characterized by Homozygous Deletions in the Multiple Endocrine Neoplasia Type I Region

Metaphase Fluorescence in Situ Hybridization Reveals Complex Rearrangements Not Detected by Conventional Cytogenetics

David Gisselsson,* Mattias Höglund,*
Fredrik Mertens,* Paola Dal Cin,[†] and
Nils Mandahl*

From the Department of Clinical Genetics,* University Hospital,
Lund, Sweden; and Center for Human Genetics,[†] University of
Leuven, Leuven, Belgium

Hibernomas are benign tumors of brown fat, frequently characterized by aberrations of chromosome band 11q13. In this study, the chromosome 11 changes in five hibernomas were analyzed in detail by metaphase fluorescence *in situ* hybridization. In all cases, complex rearrangements leading to loss of chromosome 11 material were found. Deletions were present not only in those chromosomes that were shown to be rearranged by G-banding, but in four cases also in the ostensibly normal homologues, resulting in homozygous loss of several loci. Among these, the gene for multiple endocrine neoplasia type I (*MEN1*) was most frequently deleted. In addition to the *MEN1* deletions, heterozygous loss of a second region, approximately 3 Mb distal to *MEN1*, was found in all five cases, adding to previous evidence for a second tumor suppressor locus in 11q13. (Am J Pathol 1999, 155:61–66)

Hibernomas are rare, benign tumors simulating brown adipose tissue in their histological appearance.¹ They primarily occur in the scapular, interscapular, and mediastinal regions of young adults and are cytogenetically characterized by translocations involving chromosome band 11q13, together with few or no other changes.^{2–5}

The aim of this study was to characterize in detail the 11q13 rearrangements in hibernoma by physical mapping of translocation breakpoints, using metaphase fluorescence *in situ* hybridization (FISH) analysis. Yeast and P1 artificial chromosome (YAC and PAC) probes were used for megabase-level breakpoint mapping, followed by hybridizations with cosmid clone probes for a more refined characterization.

Materials and Methods

Material from five tumors was obtained for cytogenetic analysis directly after resection (Table 1). The histopathological criteria used for classification of hibernoma have previously been described⁴; two of the cases were reviewed by the CHAMP study group.⁶ Culturing, harvesting, and chromosome banding were essentially as previously described.⁷ The cultures were harvested within 2 weeks and subcultured once or twice. Wright's stain was used for G-banding, and karyotypic descriptions were done according to ISCN (1995).⁸ The G-band karyotypes of cases 4 and 5 have previously been published by Dal Cin et al² and Mertens et al,⁴ respectively. FISH on metaphase chromosomes was according to standard protocols.⁷ Biotin- and digoxigenin-labeled whole chromosome painting probes (wcp) were purchased from Cambio (Cam-

Supported by the Swedish Cancer Society, the IngaBritt and Arne Lundberg Research Foundation, and the John and Augusta Persson Foundation for Scientific Medical Research.

Accepted for publication March 23, 1999.

Address reprint requests to David Gisselsson, Department of Clinical Genetics, University Hospital, S-221 85 Lund, Sweden. E-mail: david.gisselsson@klingen.lu.se.

Table 1. Patient Data and Karyotypes Based on G-Banding and FISH Analyses

Case no.	Age/sex	Location	Karyotype*
1	22/M	thigh	46,XY, del(11)(q13.3q13.3) , der(11)t(11;17)(q13.3;p13) , der(17)t(11;17)(q13.4;p13)/46,XY
2	58/F	shoulder	46,XX, der(9)t(9;11)(p21;q13.4) , del(11)(q13.3q13.4) , der(11)t(9;11)(p21;q13.4)/46,XX
3	41/M	shoulder	45,XY, der(13;14)(q10;q10)c/45,idem,der(2)t(2;11)(p11;q13.3) , del(11)(q13.3q13.4) , der(11)del(11)(q13.3q13.3)t(11;15)(q13.3;q22) , der(15)t(2;15)(p11;q22)
4	42/M	buttock	46,XY, der(1)ins(1;11)(p36;q13.3q13.3?5)del(11)(q13.3q13.4)t(1;2)(p36;q33) , der(2)ins(2;11)(q33;q13.3q13.4)t(2;5)(q33;q22) , der(5)t(5;11)(q22;q13.3?5) , del(11)(q13.3q13.3) , der(11)del(11)(q13.3q13.3)del(11)(q13.4)/46,idem,t(8;10)(q24;q22)/46,XY
5	47/M	buttock	46,XY, der(5)t(5;11)(q11;q13.4) , der(11)del(11)(p11)del(11)(q13.3q13.3)dup(11)(q13.3q13.4)t(5;11)(q11;q13.4) , der(11)del(11)(q13.3q13.4)del(11)(q?)dup(11)(q13.4q?)46,XY

M, male; F, female.

*Subbands were derived from hybridization results according to integrated maps in Guru et al¹¹; designations in bold type are those used in the text.

bridge) and Oncor (Gaithersburg, MD). Centromeric probes (cen) for chromosomes 5 and 11 were prepared from the α satellite clones pG-A16 and pHS53, obtained from Dr. T. Hulsebos (Amsterdam) and the American Type Culture Collection (Rockville, MD), respectively, and the 11q subtelomeric probe 2072C1 was kindly provided by Dr. L. Kearney (Oxford). YAC probes for 11q13 and the PAC clone 838L14 (Figure 1), containing *PYGM*, *GCK*, and *MEN1*,⁹ were provided by the Centre d'Etude du Polymorphisme Humain (Paris) and by D. Kedra (Stockholm), respectively. Human DNA was amplified from YAC clones by interAlu polymerase chain reaction. Cosmid probes for *PLCB3*, *PYGM*, *PPP1A*, and *MLK3* (cSOM163–8, cCL15, cCLP1A, and cMLK-3, respectively)¹⁰ were kindly provided by Dr. C. Larsson (Stockholm). *GCK*, *MEN1*, *D11S1783*, *POLA*, *D11S4933*, and *SEA* were detected with the cosmid clones 115E10, 10B11, 116B6, 33B5, 48D12, and 57H7, respectively.¹¹ Posthybridization washing was carried out in 0.4 or 1 $\times$ SSC for 2 minutes at 72°C. Counterstaining was obtained with diaminodiphenylindole. For each hybridization, 4–15 metaphase cells were analyzed. Chromosomes were identified by the diaminodiphenylindole banding patterns, complemented by cen or wcp hybridizations when necessary. Hybridizations to aberrant metaphase cells were only considered informative if signals were present on both chromosomes 11 in the cells with a constitutional chromosome complement found in the same preparation.

Results

Chromosome Banding

Clonal rearrangements involving chromosome band 11q13 were detected by G-banding in all tumors (Table 1). Cases 1 and 2 exhibited reciprocal translocations, t(11;17) and t(9;11), respectively, as the sole anomalies (Figure 2a). In case 3, with a constitutional Robertsonian 13;14 translocation, a t(2;15;11) was found, and in case 4 there were two abnormal clones, one with a t(1;11;5;2) as the sole change and a second one with t(8;10) in addition. All of these translocations were seemingly balanced. In case 5, complex rearrangements involving chromosomes 5 and 11 were seen (Figure 2b). FISH analyses

revealed additional genetic recombinations in all cases. The karyotypes based on the combined G-banding and FISH findings are presented in Table 1.

Chromosome Painting, Centromeric and Subtelomeric Probes

Hybridizations with wcp11 confirmed the translocations detected by G-banding in cases 1, 2, and 3 (Figure 2a). In case 4, material from chromosome 11 was found in all chromosomes involved in the four-way translocation: apart from the derivative chromosomes 5 and 11, it was also found in the derivative chromosomes 1 and 2, where weak, interstitial signals located at positions corresponding to the translocation breakpoints were seen. In case 5, dual-color cohybridizations of wcp5 together with wcp11 and cen5 together with the subtelomeric probe for 11q revealed complex rearrangements of both chromosomes 11 (Figure 2b). The der(11) was completely painted with wcp11, had the chromosome 11 centromere, but showed no signal with the 11q subtelomeric probe, whereas a minor part of the der(11) was positive with wcp11, cen11, and the 11q subtelomeric probe.

YAC and PAC Probes for 11q13

In cases 1–4, the YAC 897a3 hybridized proximal to the translocation breakpoints in the derivative chromosomes 11 and gave signals on the homologues not involved in the translocations (Figure 2a). The same results were obtained by hybridizations with 947h1 and 759e4 in cases 2 and 4. Similarly, in case 5, signals from 897a3 were retained on the two derivative chromosomes 11 (Figure 2b). In cases 2 and 3, 754h11 was present only on the chromosome 11 homologues. However, in case 1, the signals from the probes 754h11 and 917f10 were split by the t(11;17). In case 4, 754h11 hybridized to der(1), del(11), and der(11), and, in case 5, both signals seen were present only on the der(11). The PAC 838L14 was also split between the translocation partners in case 1 but gave no signal on the del(11). In the remaining cases, only one signal from this probe was detected in aberrant metaphase cells, which was present on the derivative

Figure 1. a: Physical map of 11q13. Human YAC and PAC clone inserts are represented by thin and thick lines, respectively, sized according to the 1000-kb bar; the broken line indicates that the insert size is unknown. YAC sizes, centiRay (cR) distances, and locations of genes and sequence-tagged sites are according to the Whitehead Institute/MIT Center for Genome Research (<http://www-genome.wi.mit.edu/>). The region commonly rearranged in the five cases is marked by a black bar. **b:** Results of hybridizations with the YAC and PAC probes. Abnormal chromosomes are according to Table 1. Symbols: —, not determined; ●, presence of one signal; ●●, presence of two signals; ○, absence of signal.

chromosomes 11 in cases 2 and 5, the del(11) in case 3, and the der(1) in case 4. The YACs 964c5 and 715d7 also yielded only one signal per metaphase cell in cases 1–3. In case 4, 964c5 was split between the derivative chromosomes 1 and 2, and was present on del(11), whereas in case 5, two signals were seen on the der(11). The distal 11q13 probes, 798d6 and 55g7, hybridized telomeric to the breakpoints in all cases. Taken together these results indicated that the 11q13 rearrangements clustered to a region covered by 754h11, 917f10, 838L14, 964c5, and 715d7, approximately corresponding to the interval between *CD5* and *D11S987*.

Cosmid Probes

In aberrant metaphase cells, two signals from the cosmid probe for *PLCB3*, located approximately 3 Mb distal to *CD5*, were present in cases 1 and 3 (Figure 2c), whereas only one signal could be observed in cases 2, 4, and 5 (Figure 3). The probe for *PYGM* yielded one signal in cells from cases 2–5 and no signals in cells from case 1, whereas the *GCK* probe gave one signal in cases 2, 3, and 5, but none in cases 1 and 4. At hybridization with the probe for *MEN1*, signals were present only in case 2, where the probe hybridized to the der(11) (Figure 2c).

Figure 2. Representative FISH and G-band images. **a:** Case 1. **Top row:** G-banding shows a seemingly balanced 11;17 translocation; **second row:** 897a3 (green) hybridizes to the del(11) and the der(11), whereas hybridization with 754h11 (red) results in signals on the del(11) and the der(17), as well as the der(11), indicating a breakpoint in the region covered by the YAC; **third row:** the der(11) and the der(17) are positive with both wcp11 (green) and 838L14 (red), but the del(11) is only wcp11 positive, indicating a cryptic deletion of the region covered by the single-copy probe; **fourth row:** hybridization with the cosmid probes for *PLCB3* (green) and *PPP1A* (red), showing a red signal only on the del(11), indicating a heterozygous deletion. **b:** Case 5. **Top row:** Complex rearrangements of chromosomes 5 and 11 shown by G-banding; **second row:** wcp5 (red) and 11 (green) also reveal rearrangement of the der(5), which is similar to a normal chromosome 11 by G-banding; **third row:** the subtelomeric probe for 11q (red) hybridizes to the der(5) and the der(11); **fourth row:** 964c5 (red) gives two signals on the der(11), and 897a3 (green) one signal each on the derivative chromosomes 11. **c:** Case 4. A normal and an aberrant metaphase cell from the same slide hybridized with cen11 (green) and the cosmid probe for *MEN1* (red) show strong signals from both probes on chromosomes 11 in the normal cell (top), but signals only from the centromeric probe in the tumor cell (bottom), although the detection threshold is set very low for this cell, as indicated by the prominent red background.

The probes for *D11S1783*, *POLA*, *D11S4933*, and *SEA* yielded one signal each in aberrant cells from cases 1–3. Because of shortage of material, analysis with these probes could not be performed in cases 4 and 5. However, the *MLK3* probe, located between *D11S4933* and *SEA*, gave one signal in cases 1–3, but two signals in cases 4 and 5; in case 5, both signals were located on the der(11), partly corroborating the duplications observed by YAC probe hybridizations. In all cases, hybridizations with the probe for *PPP1A* resulted in only one signal per aberrant cell.

Discussion

Essentially, the pattern of cytogenetic aberrations detected by chromosome banding techniques in hibernoma parallels the spectrum of rearrangements seen in lipoma, ie, one chromosome segment, 11q13 in hibernoma and 12q13–15 in lipoma, recombining with a variety of other segments.¹² In lipoma, these rearrangements frequently lead to the formation of chimeric genes involving the *HMGIC* gene in 12q15. In this study, the breakpoints in hibernoma were mapped by molecular cytogenetic tech-

niques. The results indicate that the molecular events differ distinctly from those in lipoma. Complex rearrangements of chromosome 11, leading to loss of chromosome material, were found in all five cases investigated. Deletions were present not only in those chromosomes that were shown to be rearranged by G-banding, but also, in four cases, in the ostensibly normal homologues. In these tumors, large deletions of up to 4 Mb were observed in one homologue, whereas the other one showed less extensive losses (Figure 3). The whole segment between *PYGM* and *PPP1A* was heterozygously lost in three cases, whereas two cases exhibited discontinuous, heterozygous deletions of the *PLCB3-MEN1* and the *PPP1A* regions. Such concomitant deletions in both homologues resulted in homozygous loss of *PYGM* in one case, *GCK* in two cases, and *MEN1* in four cases. The most commonly deleted segment included *MEN1* and was flanked by *GCK* and *D11S1783*, spanning a physical distance of approximately 100 kb.¹¹ To our knowledge, *MEN1* is the only gene localized to this area so far.

Germline mutations of the *MEN1* tumor suppressor gene underlie the multiple endocrine neoplasia type I (*MEN1*) syndrome, an autosomal, dominantly inherited

Figure 3. FISH results for the 11q13 region between *PLCB3* and *D11S987*. The absence or presence of cosmid signals for individual loci is indicated by open and filled ellipses, respectively. The chromosomes are referred to according to their designations in Table 1. The positions of genes and sequence-tagged sites are according to Guru et al¹¹ and references therein.

disorder with parathyroid hyperplasia, pancreatic islet cell tumors, anterior pituitary adenomas, and other neuroendocrine neoplasms.¹³ Frequent loss of heterozygosity in the *MEN1* region has been demonstrated in these tumors, in hereditary as well as in sporadic cases.¹⁴ Recent findings, however, have indicated that loss of an additional 11q13 region is implicated in the formation of *MEN1*-associated and sporadic endocrine neoplasms.^{15,16} This region was mapped between the markers *D11S4907/D11S4908* and *D11S987*,¹⁶ coinciding with the area around *PPP1A*, which was shown to be heterozygously deleted, independently of *MEN1* losses, in all five hibernomas investigated here. Thus the present study not only demonstrates the presence of *MEN1* deletions in yet another tumor type, but also provides further evidence of a second tumor suppressor locus, distal to *MEN1* in 11q13.

The finding of a high frequency of *MEN1* deletions in hibernoma touches on the question of the histogenesis of this tumor. Morphologically, hibernomas are highly similar to brown adipose tissue, and it is known that the thermogenic activity as well as the proliferation of brown adipocytes are tightly regulated by hormones such as aldosterone and melatonin in a fashion similar to that of the endocrine tissues that become neoplastic in *MEN1* patients.^{17,18} In addition, electron microscopic studies have shown that hibernomas are capable of secretory activity.¹⁹ However, if endocrine tumors and hibernomas develop along identical pathogenetic pathways, one would expect hibernomas to be part of the *MEN1* syndrome. This is not the case, however, although lipomas, angiofibromas, and other mesenchymal tumors are occasionally observed in *MEN1* patients.²⁰ Furthermore, the cytogenetic pattern of hibernomas differs considerably from that observed in *MEN1*-associated tumors: whereas

hibernomas show only a small number of changes with consistent involvement of 11q13, the few endocrine tumors analyzed so far have had mostly complex karyotypes with a number of structural and numerical changes, rarely including any chromosome 11 aberrations.¹² Thus, although hibernomas and *MEN1*-associated endocrine tumors may share some ultrastructural and pathogenetic features, including homozygous loss of *MEN1* at an early stage, it is probable that these entities subsequently progress along separate routes of tumorigenesis. This is further stressed by the fact that the benign tumors in *MEN1* patients may transform into malignancy,¹³ whereas hibernomas rarely, if ever, metastasize.¹

Apart from hibernomas, recurrent aberrations of 11q13 have been described in a number of other sporadically occurring solid tumors, eg, lipomas, astrocytomas, neurofibrosarcomas, adenocarcinomas of the breast and kidney, oropharyngeal squamous cell carcinomas, and extragonadal germ cell tumors.¹² In extragonadal germ cell tumors, the translocation breakpoints have been mapped to the segment between *PLCB3* and *MLK3*,²¹ closely corresponding to the region shown to be rearranged or lost in this study. The molecular genetic consequences, including possible deletions in the *MEN1* region, of unbalanced and seemingly balanced 11q13 changes thus appear to be of great interest for future investigations in a broad spectrum of neoplasms.

Acknowledgments

We are grateful to Prof. Settara C. Chandrasekharappa, National Human Genome Research Institute, National Institutes of Health, for critical reading and for providing cosmid clones for the *MEN1* region.

References

- Enzinger FM, Weiss SW: *Soft Tissue Tumors*, ed 2. St. Louis, Mosby, 1995
- Dal Cin P, Van Damme B, Hoogmartens M, Van Den Berghe H: Chromosome changes in a case of hibernoma. *Genes Chromosom Cancer* 1992, 5:178–180
- Meloni AM, Spanier SS, Bush CH, Stone JF, Sandberg AA: Involvement of 10q22 and 11q13 in hibernoma. *Cancer Genet Cytogenet* 1994, 72:59–64
- Mertens F, Rydholm A, Brosjö O, Willén H, Mitelman F, Mandahl N: Hibernomas are characterized by rearrangements of chromosome bands 11q13–21. *Int J Cancer* 1994, 58:503–505
- Mrózek K, Karakousis CP, Bloomfield CD: Band 11q13 is nonrandomly rearranged in hibernomas. *Genes Chromosom Cancer* 1994, 9:145–147
- Fletcher CDM, Akerman M, Dal Cin P, de Wever I, Mandahl N, Mertens F, Mitelman F, Rosai J, Rydholm A, Sciort R, Tallini G, van den Berghe H, van de Ven W, Vanni R, Willén H: Correlation between clinicopathological features and karyotype in lipomatous tumors. A report of 178 cases from the chromosomes and morphology (CHAMP) collaborative study group. *Am J Pathol* 1996, 148:623–630
- Gisselsson D, Höglund M, Mertens F, Mitelman F, Mandahl N: Chromosomal organization of amplified chromosome 12 sequences in mesenchymal tumors. *Genes Chromosom Cancer* 1998, 23:203–212
- ISCN (1995): *An International System for Human Cytogenetic Nomenclature*. Edited by F. Mitelman. Basel, S. Karger, 1995
- Kedra D, Seroussi E, Fransson I, Trifunovic J, Clark M, Lagercrantz J, Blennow E, Mehlin H, Dumanski J: The germinal center kinase gene and a novel CDC25-like gene are located in the vicinity of the PYGM gene in 11q13. *Hum Genet* 1997, 100:611–619
- Lemmens I, Merregaert J, Van de Ven WJM, Kas K, Zhang CX, Giraud S, Wautot V, Buisson N, De Witte K, Salandre J, Lenoir G, Calender A, Parente F, Quincey D, Courseaux A, Carle GF, Gaudray P, De Wit MJ, Lips CJM, Höppener JWM, Khodaei S, Grant AL, Weber G, Kytölä S, Teh BT, Farnebo F, Grimmond S, Phelan C, Larsson C, Forbes SA, Bassett JHD, Pannett AAJ, Thakker RV: Construction of a 1.2-Mb sequence-ready contig of chromosome 11q13 encompassing the multiple endocrine neoplasia type 1 (MEN1) gene. *Genomics* 1997, 44:94–100
- Guru SC, Olufemi S-E, Manickam P, Cummings C, Gieser LM, Pike BL, Bittner ML, Jiang Y, Chinault AC, Nowak NJ, Brzozowska A, Crabtree JS, Wang Y, Roe BA, Weisemann JM, Boguski MS, Agarwal SK, Burns AL, Spiegel AM, Marx SJ, Flejter WL, de Jong PJ, Collins FS, Chandrasekharappa SC: A 2.8-Mb clone contig of the multiple endocrine neoplasia type 1 (MEN1) region at 11q13. *Genomics* 1997, 42:436–445
- Mitelman F: *Catalog of Chromosome Aberrations in Cancer*, ed 6. New York, Wiley-Liss, 1998
- Miller JA, Norton JA: Multiple endocrine neoplasia. *Surgical Oncology*. Edited by RE Pollock. Dordrecht, Kluwer Academic Publishers, 1997, pp 213–225
- Debelenko LV, Zhuang Z, Emmert-Buck MR, Chandrasekharappa SC, Manickam P, Guru SC, Marx SJ, Skarulis MC, Spiegel AM, Collins FS, Jensen RT, Liotta LA, Lubensky IA: Allelic deletions on chromosome 11q13 in multiple endocrine neoplasia type 1-associated and sporadic gastrinomas and pancreatic endocrine tumors. *Cancer Res* 1997, 57:2238–2243
- Iida A, Blake K, Tunny T, Klemm S, Stowasser M, Hayward N, Gordon R, Nakamura Y, Imai T: Allelic losses on chromosome band 11q13 in aldosterone-producing adrenal tumors. *Genes Chromosom Cancer* 1995, 12:73–75
- Chakrabarti R, Srivatsan ES, Wood TF, Eubanks PJ, Ebrahimi SA, Gatti RA, Passaro EJ, Sawicki MP: Deletion mapping of endocrine tumors localizes a second tumor suppressor gene in chromosome band 11q13. *Genes Chromosom Cancer* 1998, 22:130–137
- Zennaro M-C, Le Menuet D, Viengchareun S, Walker F, Ricquier D, Lombès M: Hibernoma development in transgenic mice identifies brown adipose tissue as a novel target of aldosterone action. *J Clin Invest* 1998, 101:1254–1260
- Haldmeier G, Hoffmann K: Melatonin stimulates the growth of brown adipose tissue. *Nature* 1974, 247:224–225
- Allegra SR, Gmuer C, O'Leary GP: Endocrine activity in a large hibernoma. *Hum Pathol* 1983, 14:1044–1052
- Darling TN, Skarulis MC, Steinberg SM, Marx SJ, Spiegel AM, Turner M: Multiple facial angiofibromas and collagenomas in patients with multiple endocrine neoplasia type 1. *Acta Dermatol* 1997, 133:853–857
- Sinke RJ, Olde Weghuis D, Suijkerbuijk RF, Tanigami A, Nakamura Y, Larsson C, Weber G, de Jong B, Oosterhuis JW, Molenaar WM, Guerts van Kessel A: Molecular characterization of a recurring complex chromosomal translocation in two human extragonadal germ cell tumors. *Cancer Genet Cytogenet* 1994, 73:11–16