Parental-origin-determination fluorescence *in situ* hybridization distinguishes homologous human chromosomes on a single-cell level

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Received September 24, 2007; Accepted November 5, 2007

Abstract. The differentiation of homologous chromosomes as well as their parental origin can presently be conducted and determined exclusively by molecular genetic methods using microsatellite or SNP analysis. Only in exceptional cases is a distinction on a single-cell level possible, e.g. due to variations within the heterochromatic regions of chromosomes 1, 9, 16 and Y or the p-arms of the acrocentric chromosomes. In the absence of such polymorphisms, an individual distinction of the homologous chromosomes is not currently possible. Consequently, various questions of scientific and diagnostic relevance are unable to be answered. Based on the recently detected large-scale copy-number variations (LCV) or copy-number polymorphisms (CNP) spanning up to several mega-base pairs of DNA, in this study, a molecular cytogenetic technique for the inter-individual differentiation of homologous chromosomes called parental-origin-determination fluorescence *in situ* hybridization (pod-FISH) is presented. All human chromosomes were covered with 225 LCV- and/or CNP-specific BAC probes, and one- to five-color chromosome-specific pod-FISH sets were created, evaluated and optimized. We demonstrated that pod-FISH is suitable for single-cell analysis of uniparental disomy (UDP) in clinical cases such as Prader-Willi syndrome caused by maternal UPD. A rare clinical case with a mosaic form of a genome-wide isodisomy was used to determine the detection limits of pod-FISH. Additionally we analyzed the informativeness of conventional microsatellite analysis for the first time and compared the results to pod-FISH. With this new possibility to study the parental origin of individual human chromosomes on a single-cell level, new doors for diagnostic and basic research are opened.

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Key words: parental-origin-determination fluorescence *in situ* hybridization, homologous chromosomes, uniparental disomy, large-scale copy-number variations, copy-number polymorphisms, polymorphism

Introduction

Considering the molecular genetic level of the genome, there is a genetic variation, up to 0.1% of the DNA sequence, between any two human individuals. These differences include single-nucleotide polymorphisms (SNP), which appear in every thousand base pairs on average and are located in or outside coding regions (1), small insertion-deletion polymorphisms (INDEL) and non-coding polymorphisms like mini- and microsatellites (2). Although these DNA variations affect alleles on homologous chromosomes they cannot be used to distinguish chromosomes on a single-cell level. Differentiation of the parental origin of homologous sequences is therefore currently possible only by molecular genetic methods, such as using DNA isolated from a mixture of many different single cells, followed by approaches such as microsatellite analysis (3) or methylation-sensitive PCR (4). In contrast, conventional cytogenetics allows a differentiation with respect to maternal or paternal origin on a single-cell level, but only for a specific subset of human chromosomes and only in exceptional cases (5). This can be conducted based on size variations of the heterochromatic regions of chromosomes 1, 9, 16 and Y, or of the short arms of acrocentric chromosomes, inversion polymorphisms for chromosomes 2 and 9 (6,7) or even less frequently, if different dimensions of centromeric heterochromatin (cenh+ variants) are observed (8). In the absence of such microscopically visible polymorphisms a discrimination between human homologous chromosomes is impossible on a single-cell level (Fig. 1). Consequently, a number of questions of scientific and diagnostic relevance remain unanswered.

The picture of the human genome was remarkably changed and extended by a new kind of polymorphism discovered three years ago, called large-scale copy-number variations (LCV) (9) or copy-number polymorphisms (CNP) (10). These variations were found by DNA microarray technology and include hundreds of previously undetected structural variants in the human genome such as deletions, gains and

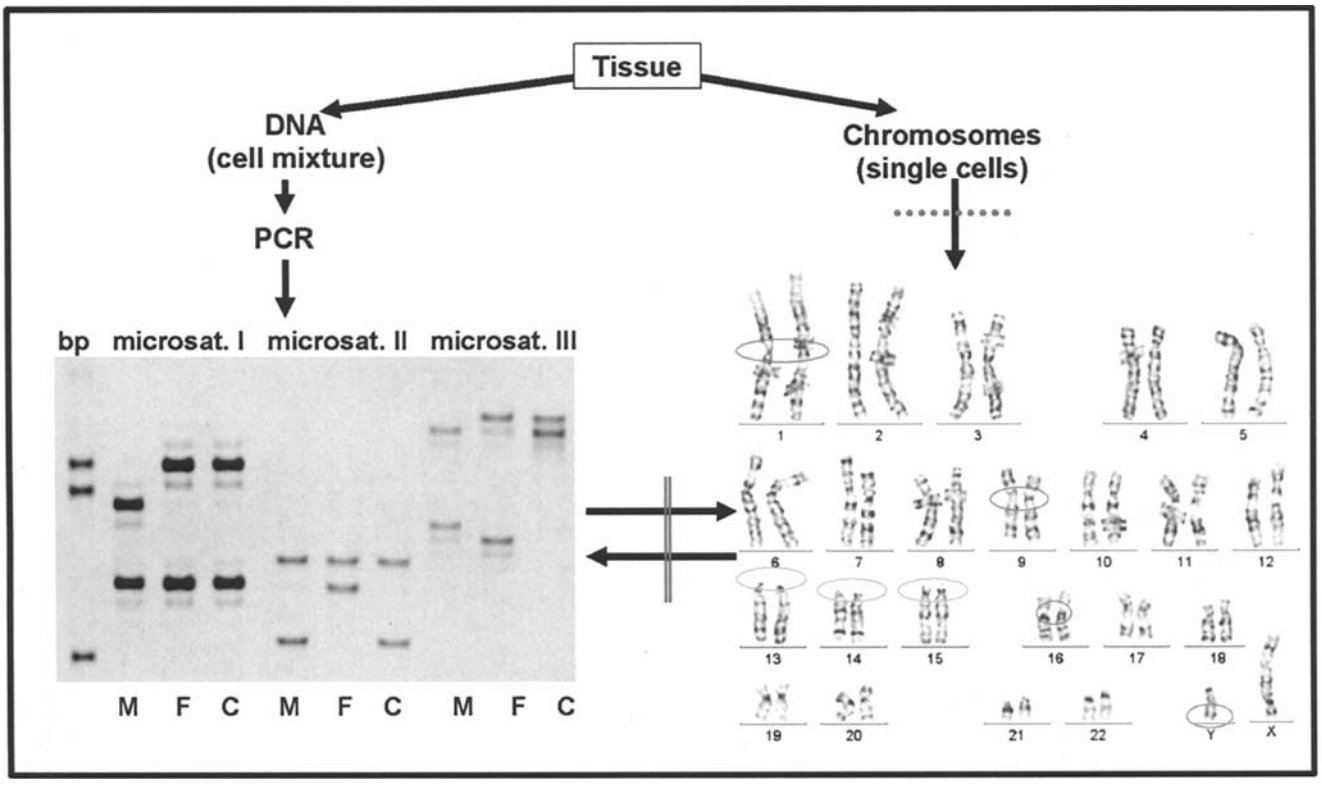


Figure 1. Two methods for distinguishing the parental origin of alleles are (left) by molecular genetic methods such as microsatellites (M, mother; F, father; C, child) based on isolated DNA from a cell mixture and (right) on a single-cell level by chromosomes that show cytogenetic polymorphisms in certain individuals and chromosomes. Yet, there is no possibility to connect the molecular genetic allele information with the homologous chromosomes and visa versa.

inversions. Surprisingly, LCV/CNP can have sizes of ten to several hundred thousand base pairs and are located in euchromatic regions all over the genome. It is expected that these large scale variations can directly affect gene dosage and therefore contribute essentially to phenotypic variation in humans (1). Currently (July 2007), there are 3643 reported structural variations (9-20) that have been collected in the database of genomic variants (http://projects.tcag.ca/variation/).

Concerning the huge size of these newly described structural variants it is currently possible to connect the molecular genetic level of DNA polymorphisms with microscopically visible homologous chromosomes. Moreover, they may be used for distinguishing cytogenetically identical homologous chromosomes. Utilizing this advantage, we describe for the first time the discrimination of homologous human chromosomes based on DNA sequence polymorphisms. This was achieved by a special fluorescence *in situ* hybridization (FISH) technique; the so-called parental-origin-determination FISH (pod-FISH). The usefulness and feasibility of this new approach is demonstrated, its efficiency will be compared to conventional microsatellite analysis and prospects for future applications are discussed.

Materials and methods

Cells. Molecular cytogenetic studies were performed on peripheral blood lymphocytes. The evaluation of pod-FISH probes was performed on chromosomes from one healthy proband. Chromosome-specific pod-FISH sets for chromosomes 1, 9 and 16 were tested on five probands showing a heterochromatin enlargement in one homologous chromosome (as described above). pod-FISH sets for all chromosomes were tested on a rare clinical case of a mosaic form of a genome-wide isodisomy. Chromosome preparations were conducted according to standard techniques (21).

Molecular cytogenetics. Two hundred and twenty-five polymorphic regions were selected which had a size >150 kb, that were reported in more than one person or study and that were reported for loss from the database of genomic variants (http://projects. tcag.ca/variation/). BAC clones were purchased from the Children's Hospital Oakland Research Institute (CHORI), Oakland, CA, USA or kindly provided by the Sanger Center. A list of all BAC clones is provided in Table I. All BAC DNA was isolated, PCR-amplified and labeled as described (22). Single and multicolor fluorescence *in situ* hybridization (FISH) techniques were performed according to published protocols (22,23).

Microsatellite analysis. Microsatellite studies were conducted in 182 UPD analyses on the basis of 299 different microsatellites (data not shown). Therefore genomic DNA was isolated from the lymphocytes of the patients, and PCR was performed as described in (24).

Results and discussion

Selection of probes and analysis of pod-FISH signals. Two hundred and twenty-five of over 3643 polymorphic loci were selected from the database of genomic variants. These were chosen, as they were reported to be frequently deleted and

Table I. Overview of BAC clones used for pod-FISH based on NCBI build 36.2.

Chr	Band	VL	BAC	AC	Start kb	End kb
1	p36.33	566	RP11-430E19	AQ552337	18	167
1	p36.33	2	RP1-283E3	AL031282	1609	1720
1	p36.22	567	RP4-636F13	AL109757	12351	12406
1	**p36.13**	**5**	**RP1-163M9**	**AL021920**	**16880**	**16998**
1	**p36.12**	**2050**	**RP11-69E9**	**AQ267525**	**22955**	**23136**
1	p32.2	9	RP6-65F20	AL138779	57301	57385
1	p31.2	10	RP11-131O15	AL358512	67446	67607
1	p21.1	326	RP11-79H19	AQ284254	102405	102563
1	p21.1	13	RP11-259N12	AL590104	103956	104113
1	**p21**	**13**	**RP11-508C1**[a]	**AL513482**	**103921**	**104016**
1	**p13.3**	**2051**	**RP11-242D10**	**AQ488595**	**108594**	**108594**
1	q21.1	1472	RP11-415J5	AQ547195	143852	143853
1	**q21.1**	**18**	**RP11-458D21**	**AL592307**	**143900**	**143901**
1	q21.1	2053	RP11-18E7	B85485	146578	146579
1	q21.1-q21.2	351	RP11-241H1	AQ481397	148067	148068
1	q25.2	21	RP11-415M14	AL162736	174297	174457
1	q31	568	RP11-109P13	BX248415	195063	195222
1	q31.3	2054	RP11-179H2	AQ419857	194526	194686
1	**q42.2**	**22**	**RP5-1016N21**	**AL139342**	**231322**	**231474**
1	q44	24	RP11-438F14[a]	AC098483	245013	245191
2	**p22.3**	**25**	**RP11-119B15**	**AC068274**	**35718**	**35886**
2	**p21.1**	**642**	**RP11-130P22**	**AC016696**	**46251**	**46406**
2	p15	27	RP11-355B11	AC016727	61513	61673
2	p12	29	RP11-398N13[a]	AC114767	82772	82779
2	p11.2	31	RP11-685N3[a]	AC096767	89040	89182
2	p11.2	29	RP11-495B16	AC109638	82554	82717
2	q21	36	RP11-32C20	AC108865	130418	130583
2	q21.1	37	RP11-89B17	AQ283656	131997	132171
2	q31.1	40	RP11-80D14	AQ283974	170976	171138
2	q35	44	RP11-316O14	AC053503	219915	220099
2	q37	46	RP11-341N2	AC093642	242709	242710
3	**p26**	**779**	**RP11-151A4**	**AQ377442**	**794**	**795**
3	p25.1	645	RP11-57D6	AQ116103	13144	13315
3	p21	47	RP11-34D21	AQ046388	60224	60224
3	p12	377	RP11-652K20	AQ407100	84963	85121
3	**q13.33**	**49**	**RP11-169N13**	**AC069444**	**120926**	**121091**
3	q26.1	52	RP11-79F11	AQ284158	165317	165467
3	q26.1	53	RP11-91B7	AQ283223	168999	169156
3	q26.32	54	RP11-114M1	AC026355	178755	178912
3	**q29**	**2056**	**RP11-245H8**	**AQ489110**	**195974**	**196140**
3	q29	56	RP11-1112O10	AQ747384	196903	197039
4	p16.3	2057	RP11-349C22	AQ543331	248	418
4	**p16.1**	**2058**	**RP11-261G12**	**AQ482925**	**6262**	**6437**
4	**p16.3**	**61**	**RP11-125L6**	**AQ344959**	**8950**	**8989**
4	p15.1	63	RP11-81N11	AQ281893	34453	34603
4	p12	572	RP11-238D1	AZ521050	45621	45630
4	q13.2	2060	RP11-279G22	AQ507098	68501	68658
4	q13.3	64	RP11-121P15	AQ351558	71415	71415
4	q25	69	RP11-18D18	B82859	112484	112501
4	q31.21	1651	RP11-412J20	AQ536199	144877	145027
4	**q32.2**	**73**	**RP11-10O3**	**AC096717**	**162954**	**163127**
4	q33	75	RP11-90E13	AQ281517	171360	171524
5	p15.33	80	RP11-812N8[b]	AQ722828	780	879
5	**p15.1**	**85**	**RP11-88L18**	**AQ281510**	**17465**	**17630**
5	p14.3	398	RP11-167E4	AQ382457	21493	21635
5	**q13.2**	**89**	**RP11-551B22**	**AC012361**	**70330**	**70517**

Table I. Continued.

Chr	Band	VL	BAC	AC	Start kb	End kb
5	q14.1	90	RP11-90A9	AQ286808	79882	80063
5	q21.1	2061	RP11-106M6	AQ317103	99184	99367
5	q21	577	RP11-346N7	AQ531580	99628	99629
5	q31.3	93	RP11-55M16	AQ082942	141030	141195
5	**q35.2**	**2062**	**RP11-259L15**	**AQ482916**	**174876**	**175053**
5	q35.3	2063	RP11-235D6[b]	n.a.	n.a.	n.a.
5	q35.3	2063	RP11-516K1	BH634779	180335	180335
6	p25.3	95	RP3-416J7	AL035696	89	214
6	p22.1	578	RP11- 111A4	AQ322113	26949	27107
6	**p21.31**	**102**	**RP3-368C2**	**AL395494**	**35624**	**35740**
6	**p21.1**	**104**	**RP3-447E21**	**AL050336**	**46035**	**46196**
6	p12.3	579	RP11-11J8[b]	B74900	47414	47569
6	**q12**	**110**	**RP3-442I1**	**AL078597**	**65020**	**65158**
6	q12	112	RP11-80L16	AQ284167	67140	67141
6	q14.1	116	RP11-897A20	AQ668040	79105	79106
6	q16.3	580	RP3-399E4[a]	AL121948	100935	100955
6	**q21**	**118**	**RP1-70A9**	**AL121788**	**109965**	**110127**
6	**q24.3**	**122**	**RP1-69B13**	**AL035698**	**146560**	**146865**
6	**q27**	**2064**	**RP11-16O10**	**B76754**	**167420**	**167579**
7	**p22**	**1704**	**RP11-460K7**	**AQ633429**	**7227**	**7420**
7	q21.1	124	RP11-79G16	AQ581376	13786	13936
7	p21.1	126	RP11-316L18	AQ507817	19069	19233
7	q11.1	339	RP11-144H20[a]	AC019063	61413	61600
7	q11.22	2065	RP11-118D11	AQ347920	66515	66674
7	q11.23	321	RP11-159N6	AQ374085	72026	72128
7	**q21.11**	**130**	**RP11-90N9**	**AQ284547**	**83058**	**83206**
7	**q22.1**	**2066**	**RP11- 204M9**	**AQ414874**	**99410**	**99593**
7	q22	430	RP11-188C21[b]	AQ417326	101711	101750
7	q22	132	RP11-577H5[a]	AC105052	101820	102007
7	q31.1	133	RP11-89O20	AQ283596	112297	112436
7	**q34**	**585**	**RP11-45N9**	**AQ195715**	**143536**	**143690**
7	**q35**	**135**	**RP4-669B10[a]**	**AC004853**	**143053**	**143082**
8	p23.3	140	RP11-159F11	AQ372530	2215	2435
8	**p23**	**440**	**RP11-774P7**	**AQ522799**	**7732**	**7917**
8	**p23.1**	**440**	**RP11-52B19**	**AQ115704**	**7873**	**7873**
8	p22	1758	RP11-366J3	AQ527596	14892	14893
8	p22	144	RP11-90I3	AQ281649	16716	16894
8	p21.3	2068	RP11-459H21	AQ580929	21181	21389
8	q21.2	445	RP11-96G1	AC023390	86851	86956
8	**q22.2**	**151**	**RP11-959D4**	**AQ742248**	**100283**	**100284**
8	q24.22	155	RP11-21H16	A084813	135975	136155
9	p24.3	158	RP11-130C19	AL136979	614	843
9	**p22.2**	**160**	**RP11-340N12**	**AL354711**	**17136**	**17299**
9	p12	594	RP11-429F11[a]	AQ550525	45232	45382
9	p12	161	RP11-93P10	AQ312901	65762	65932
9	p11.2	2071	RP11-316A6	AQ539585	42838	43023
9	p11.2	594	RP11-343E15	AQ537613	45835	45836
9	p11.2	592	RP11-433F23	AQ581745	45945	46106
9	q12	595	RP11-452D2	AL591438	67803	67803
9	q12	2072	RP11-194F8	AQ413960	68095	70113
9	q21.31	165	RP11-79G7	AQ283445	81540	81718
9	q32	169	RP11-9H12	B72555	114865	115042
9	q33.1	246	RP11-80J8[b]	n.a.	n.a.	n.a.
9	q34.3	171	RP11-413M3	AL592301	136526	136715
10	**q11.22**	**174**	**RP11-314P12**	**AL390716**	**46487**	**46562**
10	**q22.3**	**176**	**RP11-19C18**	**B82956**	**78621**	**78771**

Table I. Continued.

Chr	Band	VL	BAC	AC	Start kb	End kb
10	q22.3	2075	RP11-259A6	AQ485496	80085	80242
10	**q22.3**	**598**	**RP11-136P13**	**AQ346822**	**81551**	**81589**
10	**q23.1**	**2077**	**RP11-137H2**	**AQ382286**	**82157**	**82319**
10	q23.1	599	RP11-80I7	AQ281216	82383	82540
10	q26	340	RP11-108K14	AL161645	135079	135240
11	**p15**	**601**	**RP11-1151C19**	**AC1393737**	**4143**	**4293**
11	p15	181	RP11-168C2[a]	AC015700	10078	10257
11	**p15.1**	**2078**	**RP1-239B22[a]**	**AC124798**	**17328**	**17469**
11	**p14.3-p15.1**	**2079**	**RP11-261I18**	**AQ483542**	**21513**	**21682**
11	p11.2	185	RP11-79A4	AQ282359	48644	48801
11	**q11**	**186**	**RP11-380O22**	**AQ535337**	**55216**	**55378**
11	q12.3	189	RP11-49D19	AQ052920	62271	62448
11	q13.2	2080	RP11-280I11	AQ508928	67272	67273
11	q14.1	1860	RP11-19P3	B88382	84347	84518
11	**q14.1**	**1860**	**RP11-19P3**	**B88382**	**84348**	**84517**
11	**q22.2**	**195**	**RP11-33F6**	**AQ044970**	**102439**	**102497**
11	**q23.3**	**199**	**RP11-356E17**	**AQ535047**	**115831**	**116041**
12	p13.33	201	RP11-543P15	AC005912	3099	3265
12	p12.1	205	RP11-12D15	AC007544	22210	22369
12	**q21.32**	**209**	**RP11-900F13**	**AC024941**	**87374**	**87547**
12	q24.13	210	RP11-90D13	AQ283623	110988	111165
12	q24.33	604	RP11-146E8	AQ372302	130310	130472
13	**q21.1**	**213**	**RP11-100C24**	**AL353657**	**56600**	**56730**
13	q21.31	2083	RP11-151G10	AQ377638	62481	62844
13	**q31.1**	**216**	**RP11-80N10**	**AL136121**	**80203**	**80367**
13	**q31.1**	**218**	**RP11-417I19**	**AL162494**	**83615**	**83805**
14	q11.1	605	RP11-645B7	AQ404284	18654	18834
14	q11.1	605	RP11-831B15	AQ818166	19273	19485
14	q12	225	RP11-125A5	AQ345964	28581	28769
14	q13.3	226	RP11-26M6	B84609	34404	34592
14	q32.33	232	RP11-817G24	AQ555246	104533	104760
14	q32	232	RP11-141I7	AQ484780	105830	105831
15	**q11.1**	**233**	**RP11-138C5**	**AQ382833**	**19203**	**19367**
15	q11.1	233	RP11-2F9	B63287	19790	19970
15	q12	236	RP11-624A21	AC068448	30139	30140
15	q13	236	RP11-30N16	AC021413	30213	30213
15	q14.1	241	RP11-194H7	AQ412869	32398	32459
15	q23	2084	RP11-47G3	AQ202180	69366	69367
15	**q24.13**	**244**	**RP11-500O23**	**AZ301222**	**70607**	**70906**
15	q24	245	RP11-91O13	AQ282541	80528	81083
15	q25.2	498	RP11-246L14	AQ480297	82722	82871
15	**q26.2**	**247**	**RP11-120N1**	**AQ341084**	**95834**	**95937**
15	q26.3	2085	RP11-67J16	AQ198995	99117	99292
16	**p13.11**	**2086**	**RP11-114I21**	**AQ344890**	**15601**	**15768**
16	p13.11	2087	RP11-49G12	AQ051855	16129	16286
16	p12	250	RP11-94F6	AC133567	22200	30100
16	p11.2	2088	RP11-159J3	AQ372641	28012	28013
16	p11.2	2089	RP11-2C24	B48480	30747	30747
16	**p11.2**	**252**	**RP11-488I20**	**AC007353**	**34289**	**34491**
16	p11.2	251	RP11-96K14	AQ313535	32616	32617
16	**q12.1**	**253**	**RP11-419B13**	**AQ553383**	**48813**	**48814**
16	q22	612	RP11-142E6	AC133545	68662	68814
16	q24	255	RP11-443M9	AZ081877	84512	84607
16	q24.3	256	RP1-191P24[b]	n.a.	Subtelo 16q	Subtelo 16q
17	p12	614	RP11-721K1[a]	AC005411	13373	13538
17	q12	259	RP11-430G19	AQ552400	31949	32114

Table I. Continued.

Chr	Band	VL	BAC	AC	Start kb	End kb
17	q12	259	RP11-586K24	AQ333429	33551	33602
17	q21.2	261	RP11-29C11	B87496	36749	36924
17	q21	263	RP11-243L6	AQ488523	41572	41734
17	q23.3	267	RP11-89H15	AC025362	59472	59627
17	q24.2	2093	RP11-338N20	AQ539594	62392	62393
17	q25.3	617	RP11-762A9	AQ454240	74989	75257
17	q25.3	270	RP11-46E14	AQ201029	75316	75477
17	q25.3	2094	RP11-334C17[a]	AC015559	75616	75799
18	q12.3	274	RP11-89M10	AQ283397	37456	37620
18	q21	618	RP11-742D12[a]	AC090311	42436	42622
18	**q21.2**	**275**	**RP11-160B24**	**AQ377611**	**51861**	**52034**
18	q22	619	RP11-704G7[a]	AC021701	65892	66063
19	**p13.2**	**530**	**RP11-177L8**	**AQ412496**	**8073**	**8277**
19	p13.2	620	RP11-79F15	AQ284166	8714	8864
19	**q13.31**	**1382**	**RP11-313K22[b]**	**n.a.**	**n.a.**	**n.a.**
19	q13.31	280	RP11-21J15	AC013814	49726	49900
19	q13.33	2098	RP11-264M8	AQ486547	54767	54925
19	q13.33	2099	RP11-369N17	AQ531130	55229	55345
19	q13.4	621	RP11-155P5	AC026981	59909	59909
20	**p12.1**	**283**	**RP11-80N12**	**AQ317837**	**16723**	**16896**
20	q13.2	286	RP4-749H19	AL031674	54896	55076
21	p11.2	1394	RP11-139O21	AQ382339	10023	10187
21	q11.2	622	RP11-1126H14	AQ698240	13831	13832
21	q21.1	290	RP11-49J9	AQ053102	20982	21154
21	q22.3	293	RP11-88N2	AQ281525	43555	43770
22	q11.1	294	RP11-134C5	AQ386083	14759	14900
22	q11.21	627	RP11-379N11[b]	AQ532789	n.a.	n.a.
22	q11.2	625	RP11-775G6	AQ514830	19859	19860
22	q11.22	2011	RP11-359L2	AQ541478	21416	21417
22	q11.23	296	RP11-76E8	AQ265558	22800	22995
22	**q11.23**	**2014**	**RP11-157B2**	**AQ374326**	**23928**	**24093**
22	**q12.1**	**2104**	**RP11-259P1**	**AQ483010**	**24843**	**25024**
22	**q12.3**	**299**	**RP11-89D12**	**AQ285741**	**34601**	**34671**
X	p22.33	628	RP11-23N11	AQ014327	3862	3863
X	**p22.31**	**553**	**RP11-111F17**	**AQ341345**	**9318**	**9461**
X	p21.31	303	RP6-27C10	AL031803	29091	29143
X	p21.1	304	RP4-769D20	AL031643	33118	33253
X	**p11.1**	**305**	**RP3-323P24**	**AL022157**	**57113**	**57194**
X	**q13.2**	**306**	**RP13-36G14**	**AL139400**	**73105**	**73208**
X	**q21.1**	**309**	**RP1-223D17**	**AL121882**	**82645**	**82778**
X	q21.32	311	RP11-156J23	AL158053	92133	92310
X	**q22.3**	**313**	**RP1-75H8**	**AL158821**	**106053**	**106230**
X	q25	314	RP6-64P14	AL109800	121879	122069
X	q26	1449	RP1-119E23	Z99570	132871	132937
X	q26	2106	RP11-11I8	B71885	134067	134250
Y	p11.2	2107	RP11-35D7	AQ045270	6265	6266
Y	p11.2	636	RP11-731O16	AQ453742	9927	9928
Y	q11.2	640	RP11-135P22	AQ382241	22770	22770
Y	q11.221	2108	RP11-268K13[a]	AC022848	18627	18791
Y	q11.223	2110	RP11-355K9	AQ533417	23406	23406
Y	q11.223	641	RP11-458F2	AQ584194	23696	23861
Y	q11.223	2113	RP11-69C24	AQ236655	24578	24726

Chr, chromosome; Band, cytogenetic band; VL, variation locus; BAC, AC-accession code; Start kb and End kb, start and end of the BAC clone on the chromosome. The BAC clones that showed a polymorphism on the tested female subject are indicated in bold print. [a]Only available on NCBI 35; [b]only available on the database of genomic variants.

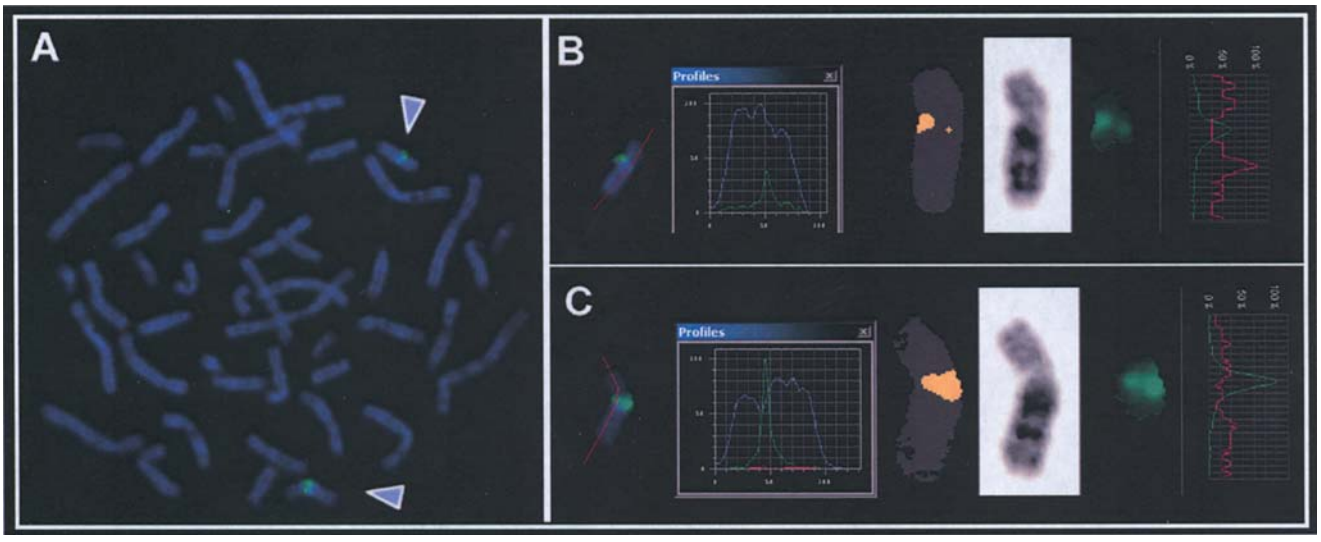


Figure 2. Example of a single pod-FISH hybridization of BAC clone RP11-488I20 (16p11.2). The signal intensity difference between the homologous chromosomes were easily detected by the naked eye on the metaphase spread (A) or by analyzing fluorochrome profiles (B, C). All examined metaphase spreads show the weaker fluorescence signal on chromosome 16 with the smaller heterochromatin block and the stronger one on the homologous chromosome with qh+ variant.

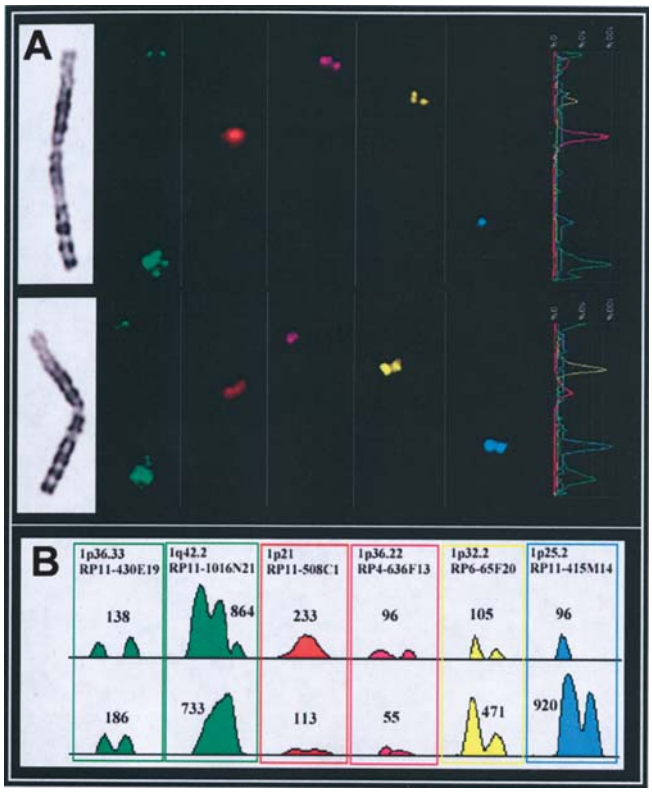


Figure 3. pod-FISH probe set for chromosome 1 showing strong signal intensity differences for the Spectrum Orange-, Cy5- and DEAC-labeled BAC clone. This was analyzed by fluorochrome profiles (A, left to right: inverted DAPI, FITC, Spectrum Orange, Texas Red, Cy5, and DEAC) and measured by SCION software (B). Values are provided for each fluorochrome channel; homologous chromosomes are placed one below the other.

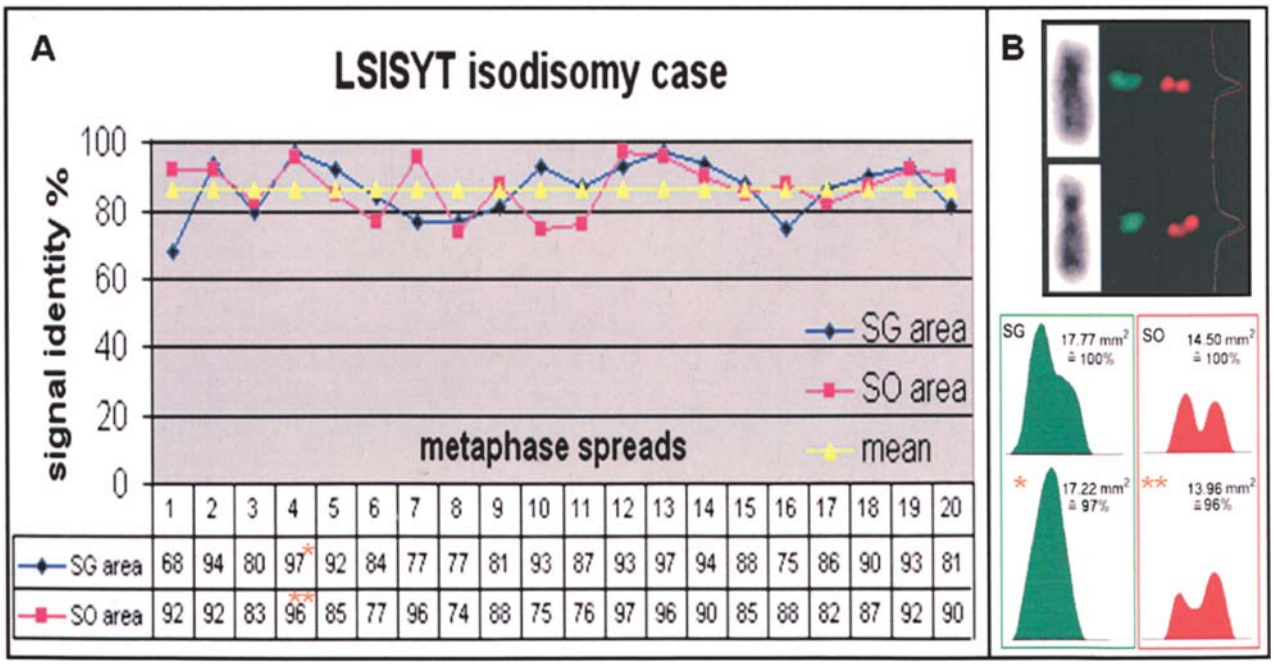
partially or completely lost. In molecular cytogenetics a loss, e.g. in microdeletion syndromes, is unambiguously detected, while a small gain of copy number, like in microduplication syndromes (23), is much harder to distinguish. Since there are no studies available on LCV/CNP frequency in the general population only regions were taken into account, that were observed in more than one person or study. Additionally, these regions needed to have a size >150 kb in order to be detectable in FISH. According to these requirements we selected 225 BAC clones from the NCBI database (http://www.ncbi.nlm.nih.gov/) as listed in Table I.

All probes were initially tested on chromosomes from one clinically healthy person. Here, the overall frequency of pod-FISH detectable polymorphisms was 29.3%; 66 of 225 regions showed a complete loss or a microscopically detectable decrease in fluorescence intensity in one of the two homologous chromosome (BAC clones in bold print; Table I).

The evaluation of pod-FISH probes is able to be performed in several ways: i) by naked eye using a fluorescence microscope (Fig. 2A), ii) by analyzing fluorescence profiles with an appropriate software (Fig. 2B and C; Fig. 3A) and iii) by measuring signal intensity and area with a software that was previously proven suitable for measuring FISH intensity signal. For our purposes, the freeware SCION (http:www.scioncorp.com) was applied (25) (Fig. 3B).

In order to observe real signal intensity differences caused by LCV/CNP polymorphisms and not variations of the FISH method itself, we analyzed 10-25 metaphase spreads per hybridization to obtain a semi-statistical mean value.

Creation and evaluation of pod-FISH sets. As it is useful to work with more than one polymorphic BAC probe simultaneously, chromosome-specific pod-FISH sets based on 5 different fluorochromes were created. For larger chromosomes such as 1, 2, 3, 4, 6, 9 and X, it was more convenient to generate chromosome arm-specific pod-FISH sets for an easier analysis and to prevent double labeling. A corresponding overview of all 31 human pod-FISH sets is provided in Fig. 5.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SG area	68	94	80	97*	92	84	77	77	81	93	87	93	97	94	88	75	86	90	93	81
SO area	92	92	83	96**	85	77	96	74	88	75	76	97	96	90	85	88	82	87	92	90

Figure 4. Cut-off assignment for FISH signal identity of non-polymorphic regions on homologous chromosomes. The example shows the values and curves for the LSISYT probe in Spectrum Orange (SO) and Spectrum Green (SG) on 20 metaphase spreads of a clinical case with a mosaic of normal and complete isodisomy. For the analysis, only isodisomic cells were chosen (A). The signal area was measured by SCION (B). The stronger signal was set to 100%. The mean identity between the signal areas of both homologous chromosomes was 86.7%. The detailed measuring is demonstrated for metaphase number 4 (*) in picture B. Fluorescent microscopic undistinguishable homologous chromosomes (top) were measured by SCION (bottom) and compared as previously described.

To test the reliability and the reproducibility of the pod-FISH approach in distinguishing homologous chromosomes, two carriers of a heterochromatic variant on one of the three chromosomes 1, 9 or 16 each were selected. pod-FISH sets as well as single probes for the corresponding three chromosomes were applied. Thus, the chromosome-specific pod-FISH intensity pattern could be correlated with the cytogenetic distinguishable homologous chromosomes. We analyzed 10-25 metaphase spreads per hybridization and found the identical, distinguishable hybridization pattern on the chromosomes with and without the heteromorphism, respectively (Fig. 2).

In order to obtain a cut-off value for differentiation between real polymorphisms and methodically caused variations within one hybridization we selected two commercially available probes (LSI SYT, 18q11.2, Abbott and LSI ABL, Abbott) and two BAC clones (RP11-358M9 and RP11-175A7) that were not located in known CNP/LCV regions. Twenty metaphase spreads per hybridization were analyzed in lymphocytes from two clinically healthy persons and one in bone marrow from a leukemia patient. Additionally, lymphocytes of a rare case of complete paternal isodisomy in 97% of blood cells was used with the above mentioned probes labeled in two different fluorochromes (Spectrum Orange or Spectrum Green) to uncover the influences of the labeling on the FISH result. In fact no microscopically visible differences were detected between the homologous chromosomes in all analyzed metaphase spreads applying the two different fluorochromes in all probands. Nevertheless, employing the sensitive SCION software we were able to measure the intensity and area of the signals on both homologous chromosomes; the more intense signal was defined as 100%. The mean value of the second signal relative to the reference signal was not <73-90% for both commercially available probes and not <74-80% for the BAC probes (Fig. 4). Therefore, we concluded that the normal variation of the FISH method for signal identity on both homologous chromosomes for a non-polymorphic region is between 10 and 36%. Thus, if in the polymorphic region the difference was >36%, a real polymorphism distinguishing both homologous chromosomes was suggested. The aforementioned experiments also demonstrated the high sensitivity of the SCION software to measure differences not visible to the naked eye (Fig. 4).

pod-FISH in UPD analysis. Imprinting of genes can cause specific syndromes when certain chromosomes are inherited from one parent only. Associated with a clinical phenotype are paternal UPD 6, maternal UPD 7, paternal UPD 11, maternal UPD 16 and maternal as well as paternal UPD 14 and 15 (reviewed in ref. 26). As described above, UPD can be investigated by microsatellite analysis of the parents and the child to determine if one parent transferred both alleles and or homologous chromosomes to the child. Therefore, we applied the pod-FISH set for chromosome 15 to a UPD case, unaware of which parent the chromosomes were inherited from, and compared the pod-FISH pattern of the parents and the child. We were able to confirm the molecular genetic microsatellite results on chromosomes investigated with pod-FISH. An example of a UPD 15 confirmation is shown in Fig. 7. It was clearly demonstrated that none of the signal patterns of the father were found in the child which could

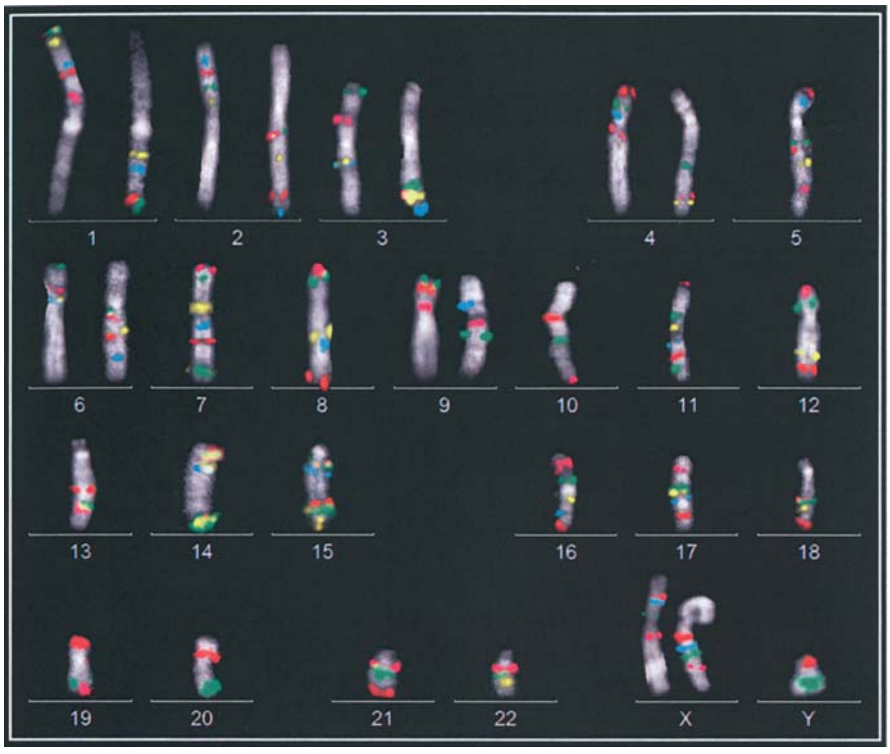


Figure 5. Overview of all 31 available chromosome-specific pod-FISH sets (independent hybridizations). For a better analysis and to prevent double labeling, we created chromosome arm-specific sets for chromosomes 1, 2, 3, 4, 6, 9 and X.

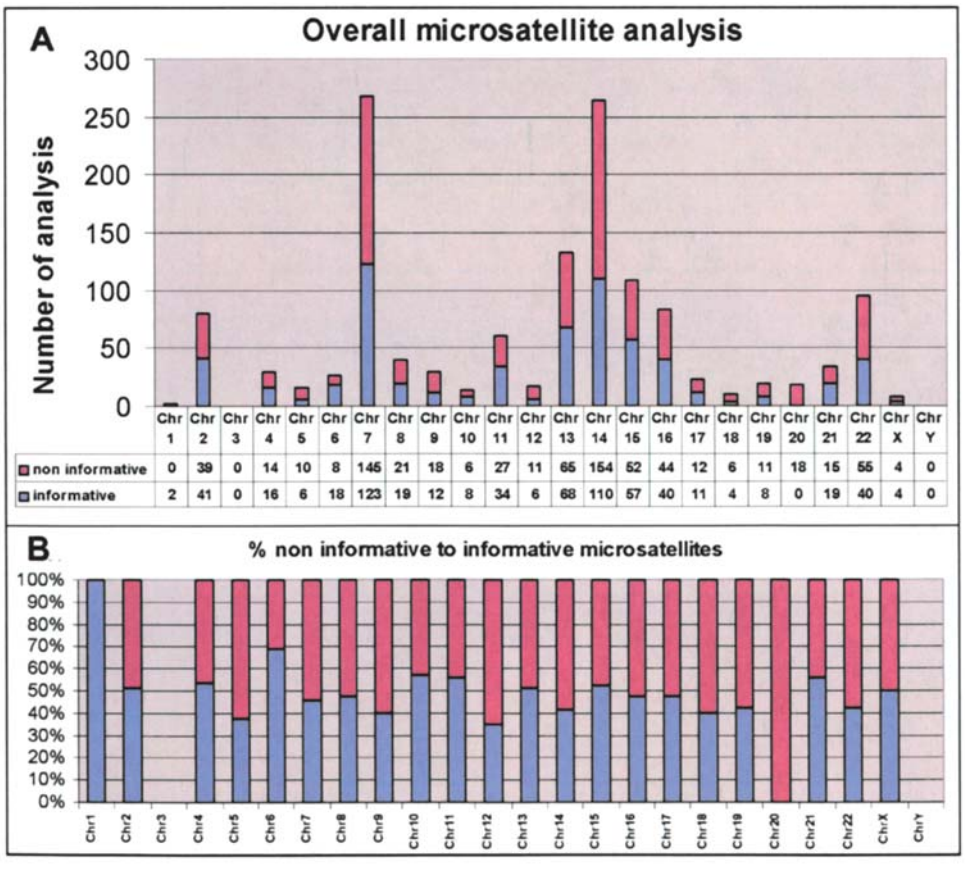


Figure 6. UPD statistics from 1545 single microsatellites showing non-informative (red) and informative (blue) results (A) in respect to the chromosomes analyzed. The overall mean value for informative situations was 47.1 versus 52.9% for non-informative situations that varied in different chromosomes depending on the number of analyzed microsatellites per chromosome (B).

Table II. Summarized results of informative situations in relation to the number of used BACs (i./n. BACs) in the 5 subjects tested for chromosomes 1 to 6.[a]

Test person	Chr 1 i./n. BACs	Chr 2 i./n. BACs	Chr 3 i./n. BACs	Chr 4 i./n. BACs	Chr 5 i./n. BACs	Chr 6 i./n. BACs
1	0/12	**2/10**	**4/8**	**1/7**	**1/6**	0/9
2	**1/12**	0/10	0/8	0/7	**3/6**	**2/9**
3	**2/12**	**1/10**	**1/8**	**1/7**	0/6	0/9
4	0/12	0/10	**2/8**	**1/7**	**1/6**	0/9
5	**1/12**	0/10	0/8	0/7	0/6	0/9
Differentiation possible in	3/5 cases	2/5 cases	3/5 cases	3/5 cases	3/5 cases	1/5 cases

[a]Differentiation of homologous chromosomes was possible for between 20 and 60% informative situations for different chromosomes and in 50% of all chromosome-specific pod-FISH sets.

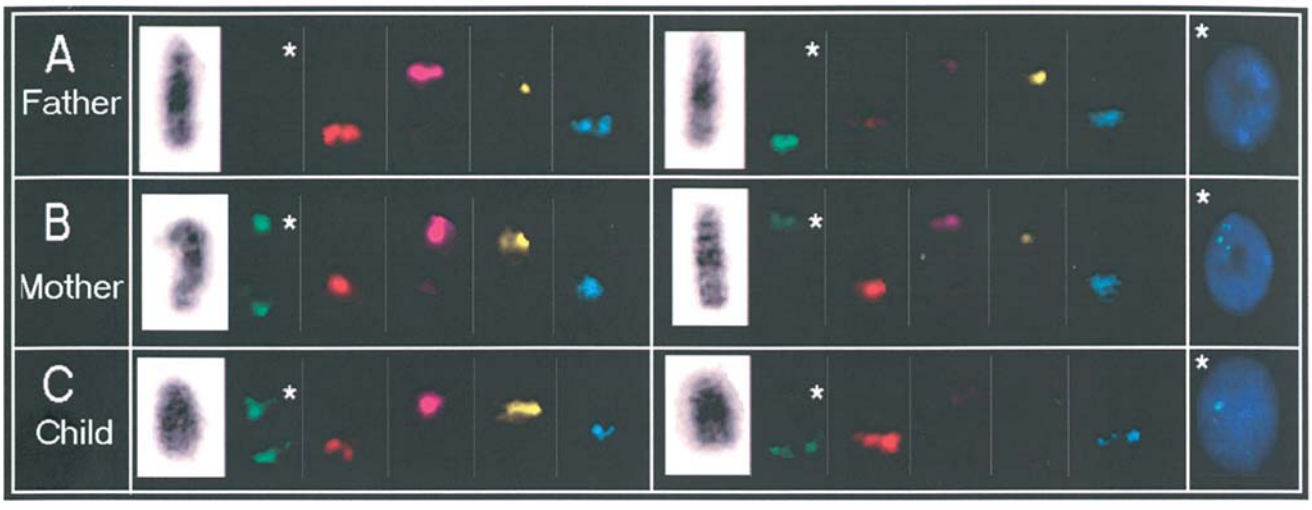


Figure 7. pod-FISH confirmation of a maternal heterodisomy 15. Comparison of the signal patterns from homologous chromosomes 15 (left to right: inverted DAPI, FITC, Spectrum Orange, Texas Red, Cy5 and DEAC) of the father (A), the mother (B), and the child (C). None of the fluorochrome compositions on both chromosomes 15 from the father fit those of the child. Yet, both fluorochrome patterns of the mother fit those of the child. *The spectrum green-labeled BAC clone RP11-138C5 in 15q11.1 that exemplifies the feasibility for interphase analysis (rightmost panels).

also be interpreted as non-paternity, but both pod-FISH patterns of the mother fit exactly to the child confirming the previously found maternal heterodisomy 15 in the child. Moreover, UPD was previously determined by microsatellite analysis (data not shown).

pod-FISH compared to microsatellite analysis. As in the well-established microsatellite analysis, the use of pod-FISH similarly can result in so-called 'non-informative' findings. This shows that the same polymorphic region on both homologous chromosomes can have the identical size and therefore are undistinguishable by the applied approach. However, there are no reports or statistics concerning the genome-wide heterozygosity frequency and/or informative situation in UPD testing by microsatellites.

In order to obtain such a dataset with which to compare the rate of informativeness of pod-FISH, the results of 177 UPD analyses by microsatellite performed in Caucasians were collected in Fig. 6. Data for all chromosomes were available apart from chromosomes 3 and Y. In summary, 1545 microsatellite PCRs on the basis of 299 different microsatellite probes resulted in 727 (47.1%) non-informative versus 818 (52.9%) informative findings (Fig. 6) that allowed a distinctive differentiation of alleles in a parent-child-trio. Therefore, approximately every second microsatellite leads to an informative situation. After evaluation of five healthy control persons for pod-FISH sets of chromosomes 1 to 6 (in summary 53 different probes in 30 experiments) a comparable rate of informativeness was observed in 20-60% of the analyzed chromosomes. In 50% of all chromosome-specific pod-FISH sets, signal intensity differences were observed (Table II). Nevertheless, a higher degree of informative LCV/CNP loci is needed to result in less non-informative situations. This can be achieved by inclusion of more BAC probes specific for other polymorphic loci in chromosome-specific pod-FISH sets. Information enabling this will be available by published CNP/LCV as more and more studies are performed studying individuals for allele

frequency estimations and *de novo* mutation rates. Currently we are working on a genome-wide high frequency CNP/LCV set with population incidences >20% based mainly on recent reports from the 270 Hap map individuals (19,20).

Future applications. pod-FISH opens a wide range of possibilites for new fields in research and diagnostics. In this study we made a first approach towards the evaluation of pod-FISH and new diagnostic areas like UPD analysis on metaphase spreads. Potential areas of use include the exclusion of maternal contamination in prenatal diagnosis, detection of chromosome homozygosity processes (e.g. in tumor genesis or aging), the determination of the origin of aberrant chromosomes, the determination of cell mixtures or mosaics (e.g. in clinical monitoring of leukemia patients), proof of paternity by chromosomes and following single chromosomes in generations by pod-FISH.

Microsatellite or SNP approaches are the method of choice to distinguish genomic DNA on a molecular level, but they are unable to determine which allele belongs to which homologous chromosome. Therefore, pod-FISH is the only method able to answer these questions. Furthermore, for molecular approaches, DNA is isolated from a number of cells that can differ in their genomic content, e.g. in cases of mosaicism. In contrast, pod-FISH analyzes single cells where mosaicism can easily be uncovered and interpreted. This can play a significant role, for instance, in AML cases where segmental UPDs were reported for chromosomes 6, 9, 10, 11, 12, 13, 15, 16, 19 and 21 that could only be detected by SNP arrays when the tumor cell fraction was >70% (27-31). Inferentially, all AMLs with a lower portion of tumor cells are not analyzable by this method although these epigenetic changes can importantly be used as tumor markers.

In conclusion, pod-FISH is the first cytogenetic method to distinguish homologous chromosomes or sequences based on submicroscopic DNA polymorphisms. The main advantage of this new technique is its performance on single cells. Furthermore, it is suitable for every chromosome. With an expected growing number of reported CNP/LCV a more dense genome-wide coverage will be achieved and more information will be available concerning single frequencies.

Acknowledgements

This study was supported, in part, by a grant from the University of Jena, the Deutsche Krebshilfe (70-3125-Li1), the INTAS (AISbl 03-51-4060), the IZKF Jena (Start-up S16), the DFG (436 ARM 17/5/06), IZKF together with the TMWFK (TP 3.7 and B307-04004), the Ernst-Abbe-Stiftung, the Stiftung Leukämie, and the Evangelische Studienwerk e.V. Villigst.

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