Combined mutation and CNV detection by targeted next-generation sequencing in uveal melanoma

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Abstract

Uveal melanoma (UM) is a highly aggressive cancer of the eye, in which nearly 50% of the patients die from liver metastasis. It is the most common type of primary eye cancer in adults. Chromosome and mutation status have been shown to correlate with the disease free survival. Loss of chromosome 3 and inactivating mutations in *BAP1*, which is located on chromosome 3, are strongly associated with ' high risk' tumors that metastasize early. Other genes often involved in UM are *SF3B1* and *EIF1AX*, which are found to be mutated in intermediate- and low risk tumors, respectively. We developed a targeted sequencing method that can detect mutations in genes involved in UM and chromosomal anomalies in chromosome 1, 3 and 8. Whereas current UM-diagnostics involves several techniques for detection of copy number variations and somatic mutations, our targeted UM panel can detect losses and gains of chromosome 1, 3 and 8 and somatic mutations in the aforementioned genes in a single assay. By sequencing 27 formalin-fixed paraffin-embedded and 43 fresh UM-specimens, we show that mutations and chromosome-status can reliably be obtained using targeted next generation sequencing (NGS).

Introduction

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults with a worldwide annual incidence in Caucasians of 5-7 per million per year[1]. Despite successful treatment of the primary tumor, nearly 50% of the patients develop liver metastasis within 5 years. Once metastatic disease is diagnosed, survival is between 2 and 9 months[2]. Approximately 40% of UM patients develop metastases within 4 years, but dissemination can occur even up to 4 decades after diagnosis[3]. This demonstrates that the prognosis for UM patients can strongly vary between patients, and is dependent on a number of factors, including clinical and histological parameters, as well as the underlying genetic 'make up' of the tumor cells[4].

Chromosomal anomalies are often found in solid tumors, but previous work has shown that most of the chromosomal anomalies in UM are limited to chromosome 1, 3, 6 and 8. Some of these chromosomal variations correlated with metastasis, such as loss of chromosome 3[5]. Monosomy 3 is observed in half of

the UM patients and is strongly associated with poor survival. It is thought to be an early event, since it is often accompanied by other chromosomal anomalies, such as gain of chromosome 8q[6,7,8]. Another common anomaly in metastasizing UM with monosomy 3 is loss of chromosome 1p[9]. Chromosome 6 shows frequent rearrangements in both p- and q-arm in UM; yet, deletion of 6q or gain of 6p are not associated with metastatic disease[10].

UM are genetically well-characterized tumors. Recent research using genome-wide sequencing led to the discovery of several genetic alterations in UM, which correlate to a distinct survival pattern. Activating mutations in guanine-nucleotide binding protein- Q (*GNAQ*) and -alpha 11 (*GNA11*) were found in the majority of UM patients (93%), and are therefore thought to be initiating mutations[9,10.11]. Inactivating mutations in the BRCA-associated protein 1 (*BAP1*), located on chromosome 3p, were found in the early metastasizing patients[14]. Recently two other genes have been reported that play a role in UM biogenesis. Mutations in the eukaryotic translation initiation factor 1A (*EIF1AX*) were observed in non-metastasizing tumors[15] and a hotspot mutation in the splicing factor 3 subunit 1 (*SF3B1*)-gene was detected in late metastasizing tumors[16]. Both of these genes are known to be mutually exclusive.

Current clinical diagnostics for UM include several techniques, such as copy number analysis by single nucleotide polymorphism (SNP)-array[17], multiplex ligation-dependent probe amplification (MLPA)[18] or fluorescence in situ hybridisation (FISH)[19], immunohistochemistry of the BAP1 protein[20,21] and Sanger sequencing of *EIF1AX*, *SF3B1* and *BAP1*. In some cases, whole genome sequencing (WGS) or whole exome sequencing (WES) is used to identify the somatic mutations present in the tumor. In this study we performed Ion Torrent next generation sequencing (NGS) with a custom made NGS panel on 70 UM to determine if targeted sequencing can be implemented in the routine UM-diagnostics. This panel has been designed specifically for UM, covering all major hotspot mutations in the five relevant UM-genes and several SNPs on chromosome 1, 3 and 8 to allow analysis of clinically relevant chromosomal anomalies.

Methods

UM samples

Sixty-five UM samples were selected from our Rotterdam Ocular Melanoma Study Group (ROMS)-database and 5 were external samples from patients who underwent enucleation for UM, received for diagnostics from the Liverpool Ocular Oncology Research Group (LOORG). Samples included in this study were diagnosed as UM, collected between 1988 and 2016, and include formalin-fixed paraffin-embedded (FFPE) and fresh specimens. A written informed consent was obtained before treatment, the study was performed according to the guidelines of the Declaration of Helsinki and was approved by the local ethics committee.

DNA extraction

Targeted NGS was performed on DNA extracted from fresh- and FFPE samples. For all tumor samples, an ophthalmic pathologist reviewed and selected tumor areas with an estimated minimal tumor cell percentage of 85%. DNA isolation from fresh tissue was carried out using the QIAmp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. For FFPE samples, depending on the size of the tumor, 2-6 5µm FFPE sections were deparaffinized and hematoxylin stained prior to isolation of the DNA. FFPE tumor tissue was microdissected by scraping the cells manually from hematoxylin-stained sections. DNA was then extracted by incubation of the tissues overnight at 56°C in lysis buffer (Promega, Madison, Wisconsin, USA), containing 5% Chelex (Bio-Rad, Berkley, California, USA) and Proteinase K (Qiagen). Proteinase K was inactivated by incubating the sample for 10 minutes at 95°C and cell debris was pelleted down together with the Chelex by centrifugation in a microcentrifuge at maximum speed. DNA concentrations were measured with the Quant-iT Picogreen assay kit (Thermofisher Scientific, Grand Island, New York, USA), as described by the manufacturer. All DNA samples were stored at -20°C. The DNAs provided by the LOORG had been extracted as previously described using the Qiagen DNeasy Blood and Tissue kit[2].

Targeted Next-Generation Sequencing

A custom primer panel covering the five UM genes and several SNPs located on chromosomes 1, 3 and 8, was designed using Ion Ampliseq Designer 2.0 (ThermoFisher Scientific). This resulted in an 11.5 kb amplicon panel, containing 98 amplicons. Amplicons designed for *GNAQ*, *GNA11*, *EIF1AX* and *SF3B1* covered only the exons containing the known mutation hotspots. All exons of the *BAP1* gene were covered by amplicons. On chromosome 1 and 8, seventeen amplicons were designed to cover highly polymorphic regions in the entire chromosome (Supplementary table 1). These highly polymorphic regions with a global minor allele frequency of at least 45% were selected based on data found in the NCBI SNP database[23]. For chromosome 3 twenty-one amplicons were designed, due to the clinical relevance. The DNA input varied between 3 and 10 ng, depending on the amount of DNA available per sample. Library construction was performed using the AmpliSeq Library Kit 2.0. Next-Generation amplicon sequencing of the libraries was performed by semiconductor sequencing with the Ion Torrent Personal Genome Machine (PGM) (Thermofisher Scientific) on an Ion Chip, according to the manufacturer's protocol.

Mutation Analysis

Raw Ion PGM sequence data was analysed using Torrent Suite Software V4.4.3 (Thermofisher Scientific) with Variant Caller v3.6 and Coverage Analysis plugins to identify variants and perform sequence coverage

analysis, respectively.

Sanger sequencing

DNA from 23 tumor samples was sequenced using the Sanger method to confirm results found by NGS. Selected regions of the genes of interest were amplified by polymerase chain reaction (PCR). Subsequently, sequencing of the PCR products and mutation analysis of *GNAQ*, *GNA11*, *BAP1* and *SF3B1* and *EIF1AX* was done as reported previously[13,16,20]. Alignment of the sequence reads was done with reference sequence Hg19 from the Ensemble genome database.

Immunohistochemical staining

To detect loss of the BAP1 protein in tumors, immunohistochemical staining of BAP1 was performed on 4µm FFPE sections of tumors. Staining was done by an automated immunohistochemistry staining system (Ventana Medical Systems Inc, Tucson, Arizona, USA) as described before[20]. BAP1 protein expression data were also available for the cases received from LOORG, which were stained as previously described[24]. Sections were evaluated by the ophthalmic pathologists in Rotterdam and Liverpool (RV and SEC, respectively).

Copy number variation analysis

Validation of the copy number status of the chromosomes was performed by SNP-array, MLPA and FISH analysis. Two hundred nanograms of fresh tumor DNA was used for the Illuminia 610Q SNP-array (Illumina, San Diego, California, US). Results were analyzed with Nexus Software (BioDiscovery, El Segundo, California, USA). One hundred nanograms of DNA from each FFPE UM was used for MLPA analysis of chromosomes 1p, 3, 6 and 8 as previously described20. FISH analysis was performed on directly fixed tumor material, with probes for chromosome 1, 3 and 8 as reported previously[19]

Results

Coverage of UM genes

To detect mutations in the *GNAQ*-, *GNA11*-, *EIF1AX*-, *SF3B1*- and *BAP1* gene, 43 amplicons were used to sequence these genes reliably. Samples with a minimum total read count of 40.000 were analyzed for mutations in the five UM genes. The total amount of read counts for fresh samples was on average slightly higher than those of FFPE samples (Fig.1a). Most of the amplicons covering the five UM genes consisted of 1 – 2% of the total read count, which corresponds to a minimum of 400 reads (Fig.1b). The median read count of all amplicons was 1,3%. Several amplicons obtained a coverage of less than 1% of the total read count, such as *EIF1AX* exon 1 and *BAP1* exon 1 and 3. By adding extra amplicons in the primer mix for these areas, we compensated for these lower read counts.

Mutation analysis

Seventy UM samples were sequenced with our targeted UM panel. DNA was isolated from fresh specimens (n=43) and from FFPE-material (n=27). From all 70 samples sufficient DNA was extracted for sequencing. Forty-one percent of the samples harbored a *GNAQ* exon 5 p.Gln209Pro or p.Gln209Leu mutation, 3% a *GNAQ* exon 4 p.Arg183Gln mutation, 41% a *GNA11* exon 5 p.Gln209Leu mutation, 1% a *GNA11* exon 4 p.Arg183Cys mutation and in the remaining samples no mutations in either of these two genes were detected (Table 1). Mutations in the *BAP1* gene were found in 41% of the cases, mutations in *SF3B1* in 16% and *EIF1AX* in 20% of the samples (Supplementary Table 2). We validated the results in 40% of the samples by Sanger sequencing and identified no new mutations in either of these genes. The obtained results do not entirely overlap with the known mutation rate for UM, but those differences can be explained by the bias in our sample population.

Detection of loss of BAP1 protein expression

Absence of the BAP1 protein is often associated with monosomy 3 UM. The loss of nuclear BAP1 expression can be immunohistochemically assessed, which is routinely performed in a diagnostic setting. UM samples were sequenced and analysed for *BAP1* mutations. The results obtained from three samples are depicted in figure 2; one sample with a normal BAP1 expression and two samples with loss of BAP1 expression. HE staining indicated a high presence of tumor cells in all three samples (Fig. 2a). BAP1 staining was positive for the upper sample and negative for both the middle and lower sample (Fig 2b). Ion Torrent sequencing of the *BAP1* gene revealed no mutations in the top sample but did show a 5 basepair deletion and insertion in exon 4 of *BAP1* in the middle sample, resulting in a frameshift and a stop(p.Arg59Lysfs*12). The lower sample harboured a nonsense mutation in exon 6 (p.Glu136*) (Fig 2c), confirming the presence of *BAP1* mutations in the IHC BAP1 negative tumors. BAP1 IHC was carried out for 59 samples. In 8,4% of these cases the BAP1 IHC did not correspond to the mutation status and copy number of chromosome 3, found by Ion Torrent sequencing of the *BAP1*-gene (Supplementary table 2). However, for the majority of the samples the UM panel can correctly detect mutations corresponding to the observed loss of BAP1-expression.

Copy number analysis

SNP, MLPA and FISH analyses are commonly used to identify chromosomal changes in UM tissues. To determine whether the Ion Torrent UM Custom panel allows a reliable detection of allelic imbalances caused by (partial) losses and gains of chromosome 1, 3 and 8, we compared results obtained by FISH and SNP-array with the CNV results from our custom UM panel. SNP covering amplicons were evenly distributed over the entire chromosome (Fig.3a), which allowed us to observe partial aberrations as well. FISH results of the upper sample in figure 3 shows two signals for chromosome 3 (red signal) and the control FISH probe on chromosome 5 (green signal). The lower sample shows a loss of chromosome 3 (Fig.

3b). This was confirmed with the SNP array, where the Log R Ratio and B-allele frequency shows no loss of heterozygosity for chromosome 3 in the upper sample and monosomy 3 for the lower sample (Fig 3c). The same pattern of allelic distribution was seen with the Ion Torrent SNP-analysis of chromosome 3 (Fig. 3d). The B-allele frequencies for chromosome 1 and 8 were confirmed as well, as shown in supplementary figure 1. Across all samples we found that 52% showed monosomy 3, 30% loss of chromosome 1p and 57% gain of chromosome 8q. These percentages overlapped with the percentages found by other CNV-techniques, such as SNParray, MLPA and FISH analysis. (Supplementary table 3).

Discussion

UM is characterized by recurrent mutated genes and chromosomal anomalies. In this study we present a novel custom-designed NGS assay for UM, which can be used to predict UM patients' prognoses based on mutation status and chromosome status of chromosome 1, 3 and 8. The assay can be conducted with using either freshly-isolated DNA or DNA obtained from FFPE material. This is the first study that establishes a method that can be used for UM diagnostics on both FFPE and fresh UM material. Our assay is cost-effective, since one method can replace several other techniques, such as FISH, SNP-array, BAP1 IHC and Sanger sequencing. Other important advantages are the low amount of DNA (10 ng) necessary for sequencing and the small amplicon-size, which makes this technique suitable for degraded FFPE material.

Prognostication of UM patients can be achieved by analyzing mutation status. Currently, this is usually performed by Sanger sequencing. Mutations in *GNAQ*, *GNA11* and *SF3B1* occur almost exclusively in hotspot locations, therefore only these locations have to be sequenced. Since mutations can occur throughout the entire *BAP1* gene, large amounts of DNA are needed for sequencing of multiple exons. Whole exome-sequencing (WES) is a reliable and easy method to obtain mutation status as well. However, since only a few genes are involved in the oncogenesis of UM, many irrelevant reads will be produced. WES is less cost-effective for the diagnostic setting, compared to targeted Ion Torrent sequencing.

Several regions of the human genome are difficult to cover with NGS. As shown in figure 1b, a few exons, such as *BAP1* exon 1 and the first two exons of *EIF1AX*, show a relatively low read count. Due to this low read count, it is more difficult to detect mutations in this particular exon. These findings are not only observed in our targeted UM panel, but also in Whole Genome sequencing data of UM[25,26]. Since exon 1 of the *BAP1* gene is located in the non-translated region, the effect of a mutation in this UTR region is not always clear. Another region, which is sensitive for sequencing errors is exon 1 of *EIF1AX*, caused by a pseudogene on chromosome 1. Amplicons covering only exon 1 may also produce reads derived from chromosome 1. By adding a second set of reads generated by a different amplicon for *EIF1AX,* we now cover not only exon 1 but also a part of the 3'UTR, which will obtain longer reads that can only be derived

from *EIF1AX* exon 1.

Besides mutation status-analysis, our UM panel also provides information about the copy number status. Techniques such as FISH, MLPA and SNP-array can provide information about the chromosomal change of one or several chromosomes in the tumor in most cases, but these techniques also have their disadvantages. The probes used for FISH are specific for a certain region, i.e. FISH testing does not screen the entire chromosome. It is also a relatively laborious technique, which can take up to several days. Performing a SNP array requires less time, but the amount of DNA necessary (200 ng) is significantly higher than other techniques. Furthermore, standard SNP analysis is less successful on DNA extracted from FFPE tissue compared to -freshly obtained DNA. With our UM panel, we reliably detect CNVs by NGS of highly polymorphic SNPs. Since this assay requires less DNA than conventional SNP-arrays and less time than FISH, it is a promising method for routine UM diagnostics. The SNP analysis performed with this UM panel does not allow detection of polyploidy in UM samples. However, recently it has been shown that polyploidy in UM does not change the mutation prevalence, which means that detecting polyploidy in UM patients has little impact in this method since it does not affect the prognosis[27].

Our Ion Torrent UM panel is in the current state already suitable for implementation in UM prognostication, with the advantage that it can easily be expanded by adding the more recently discovered UM genes into our panel. Recently, it has been reported that a small percentage of the UM samples contain mutations in other spliceosome components, *SR2F2* and *U2AF1*. It is thought that these tumors act in the same way as *SF3B1* mutated tumors. Other rare alterations in UM tumors are mutations in *PCLB4* and *CYSTLR2*, which are downstream targets of GNA11 and GNAQ and are thereby thought to be less suitable for prognostication[28].

In summary, we present a NGS-based assay that can readily be implemented as a diagnostic pathology application for UM. Mutation and CNV data can be obtained by one technique, which can establish a reliable diagnosis for UM patients. At present there is no successful treatment for metastasized UM; however, with the development of new therapies, identification of high-risk UM patients will be very important, particularly in adjuvant therapy trials. Our custom-designed UM panel will make a valuable contribution to the rapid stratification of UM patients.

Acknowledgements

We thank Quincy van den Bosch for his assistance with imaging and Tom de Vries-Lentsch for helping with the figures. This study was supported by a grant of the Combined Ophthalmic Research Rotterdam.

Disclosure/Conflict of interest

The authors declare no conflict of interest.

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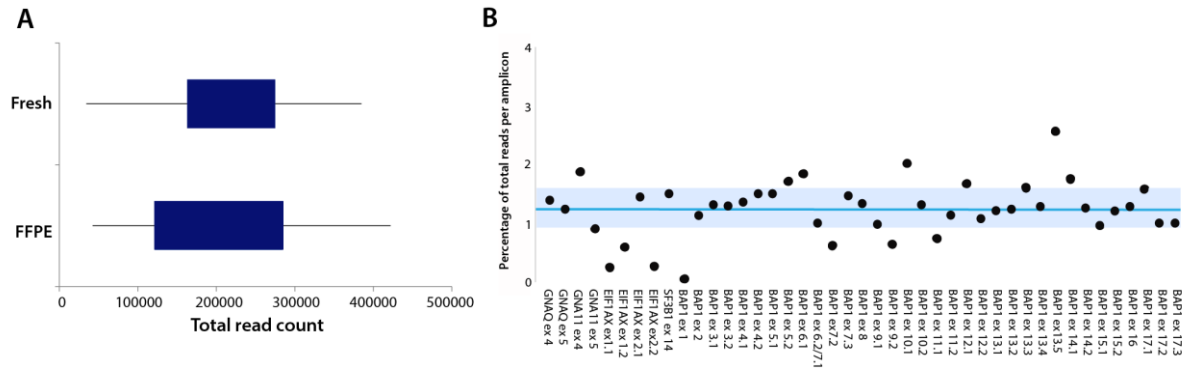


Figure 1. Sequencing efficiency of FFPE and fresh UM specimens

A) Boxplots showing the total read count for all fresh- (top plot) and FFPE samples (bottom plot) B) Percentage of total reads visualised for all amplicons covering the five UM-genes. Blue line indicates median for all amplicons and light blue area shows second- and third quartile.

Table 1. Amplicon location and mutation rate for the five genes relevant in UM

Gene	Chromosome	Exons	Codons	Mutation rate (%)
GNA11	19	4, 5	183, 209	41
GNAQ	9	4, 5	183, 209	49
EIF1AX	X	1, 2	4 – 44	21
SF3B1	2	14	1873, 1874	16
BAP1	3	1 - 17	1 - 730	43

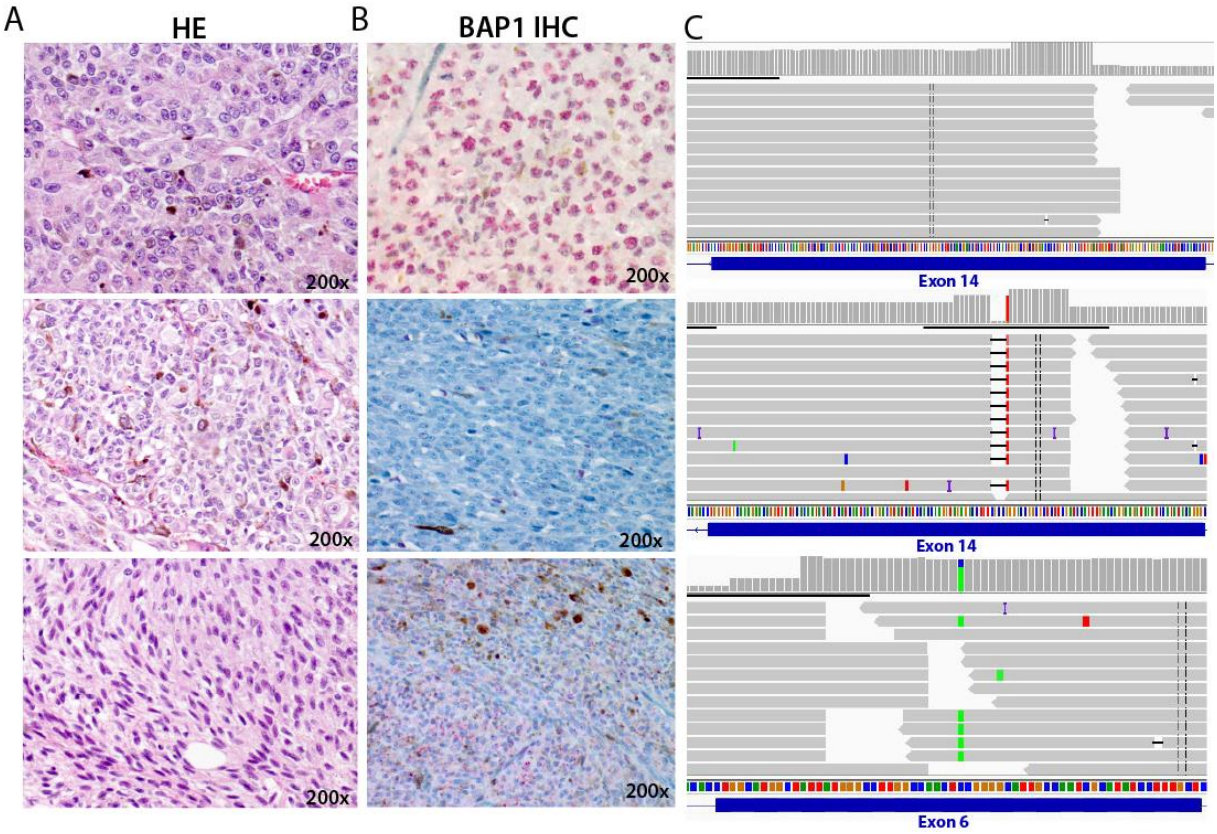


Fig 2. Histopathological and genetic aspects of three uveal melanoma specimens
A) HE-staining of three UM samples (200x) B) IHC staining of BAP1 protein showing strong nuclear BAP1 expression in the top sample and loss of BAP1 expression in middle and bottom sample (200x) C) From top to bottom: no mutation observed in the BAP1-gene, a 5-basepair deletion and insertion in exon 4 resulting in a frameshift ((p.Arg59Lysfs*12) and a point mutation in exon 6 which changes a Glutamate into a STOP-codon (p.Glu136*) .

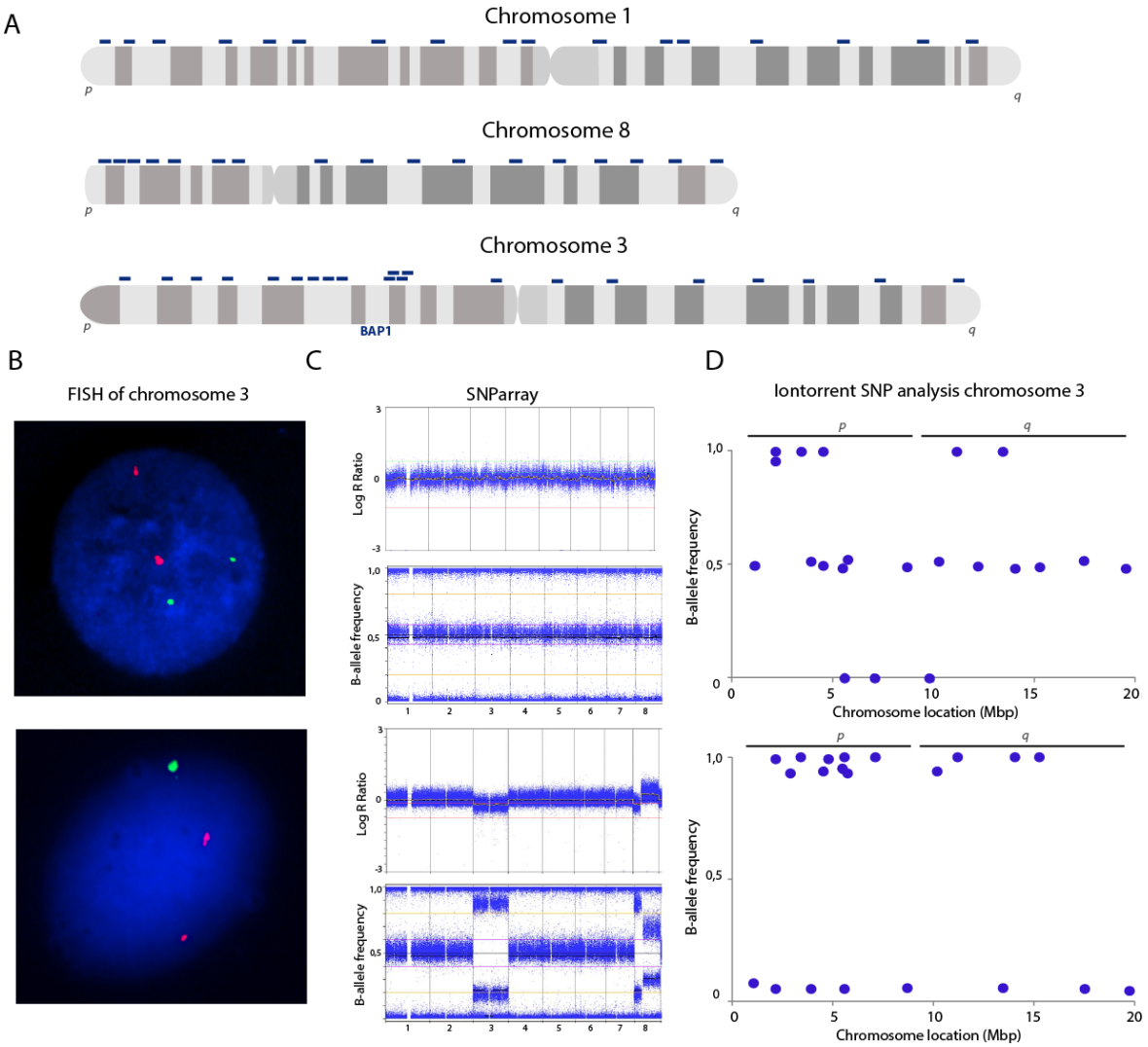


Fig 3. Copy number analysis of chromosome 3

A) Visualisation of the evenly spread amplicons covering highly polymorphic SNPs on chromosome 1,3 and 8. **B)** FISH of chromosome 5 (red) and chromosome 3 (green) shows no loss for chromosome 3 in the top sample and loss of chromosome 3 in the bottom sample **C)** Top SNP array visualises chromosome status for chromosome 1 to 8. Both Log R Ratio and B-allele frequency indicate disomy 3, whereas the SNP array for the bottom panel shows loss of chromosome 3 **D)** SNP analysis done by the targeted UM panel visualises the B-allele frequency for chromosome 3. Top SNP analysis shows heterozygosity for the SNPs, indicating disomy 3, while bottom sample shows no heterozygous variants indicating loss of heterozygosity of chromosome 3.

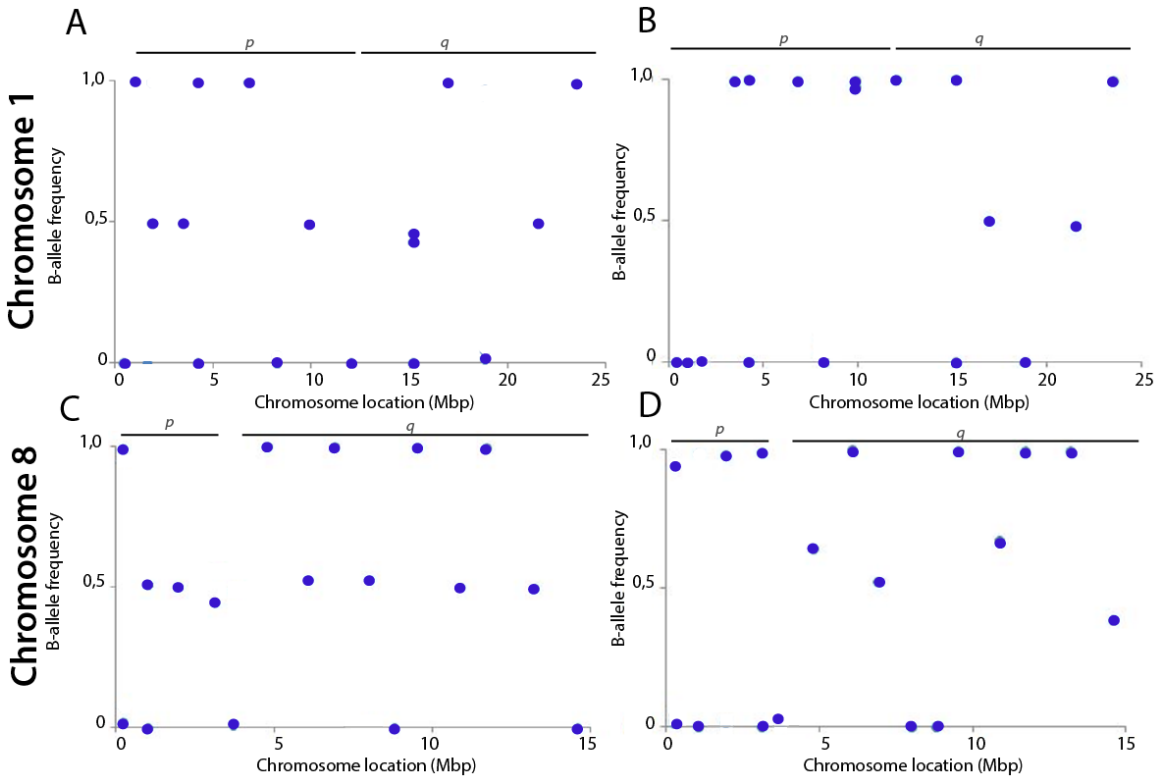
Supplementary table 1. List of the highly polymorphic SNPs covered by the UM panel

	SNP-number	Position (bp)
Chromosome 1	rs7418256	4,084,304
	rs7412149	9,579,964
	rs12048851	16,382,718
	rs10907287	18,497,478
	rs6425861	34,372,503
	rs639298	42,001,530
	rs11209106	68,001,206
	rs480304	82,123,485
	rs10493903	98,900,818
	rs17258467	120,323,058
	rs1752380	151,347,746
	rs3856201	163,736,341
	rs10753786	169,288,770
	rs2072040	175,096,333
	rs138685314	188,228,295
	rs6681013	215,154,797
	rs592197	234,817,283
Chromosome 3	rs1601368	10,829,535
	rs1549356	21,528,837
	rs7612272	28,816,226
	rs7648156	34,497,918
	rs1274960	39,192,542
	rs267218	45,633,834
	rs9311387	46,115,590
	rs295449	47,375,955
	rs3821659	54,987,923
	rs2702143	55,738,509
	rs9868630	56,012,096
	rs62259027	57,747,389
	rs9310190	70,420,837
	rs12497448	86,741,603
	rs1151334	102,257,506
	rs3749299	111,673,147
	rs4045771	121,962,478
	rs975149	134,666,475
	rs1004009	152,754,481
	rs9866779	175,021,665
	rs11717776	197,569,559
Chromosome 8	rs2405488	2,141,263
	rs4498602	10,180,242
	rs17577614	15,470,729
	rs13275706	19,327,151
	rs6557699	23,602,610
	rs1882928	31,023,822
	rs10095600	36,911,156
	rs4147426	47,909,945
	rs10107875	60,526,565
	rs6995640	68,904,187
	rs2120410	79,844,006
	rs13261311	87,705,504
	rs4735258	94,935,937
	rs4734993	108,686,209
	rs2142250	117,093,062
	rs6415522	131,905,690
	rs7008457	145,536,593

Supplementary table 2. Mutation status, BAP1 IHC and chromosome 3 status of all 70 samples

	Tissue	GNAQ ex 4	GNAQ ex 5	GNA11 ex 4	GNA11 ex 5	EIF1AX ex 1/2	SF3B1 ex 14	BAP1	BAP1 IHC	Monosomy 3
UM-1	FFPE		●ˢ			●ˢ			+	
UM-2	FFPE		●					●	-	●
UM-3	FFPE		●					●	+/-	
UM-4	FFPE				●			●	ne	●
UM-5	FFPE		●					●	ne	●
UM-6	FFPE		●					●	ne	●
UM-7	FFPE				●		●		ne	
UM-8	FFPE		●			●			ne	
UM-9	FFPE								+	●
UM-10	FFPE				●				+	●
UM-11	FFPE				●		●		+	
UM-12	FFPE				●		●		+	
UM-13	FFPE				●		●		+	
UM-14	FFPE				●				+	
UM-15	FFPE				●				+	
UM-16	FFPE				●				+	
UM-17	FFPE				●			●	-	●
UM-18	FFPE		●			●			+	
UM-19	FFPE				●			●	+	●
UM-20	FFPE							●	-	●
UM-21	FFPE				●		●		+	
UM-22	FFPE				●		●		+	
UM-23	FFPE		●					●	-	●
UM-24	FFPE				●		●		+	
UM-25	FFPE				●ˢ			●ˢ	+/-	●
UM-26	FFPE		●ˢ						+	
UM-27	FFPE				●ˢ			●ˢ	-	●
UM-28	Fresh								Ne	
UM-29	Fresh		●ˢ					●ˢ*	+	●
UM-30	Fresh					●			+	
UM-31	Fresh				●	●			+	
UM-32	Fresh								+/-	
UM-33	Fresh		●			●			+	●
UM-34	Fresh								+	
UM-35	Fresh				●ˢ	●ˢ			+	
UM-36	Fresh		●					●	-	●
UM-37	Fresh		●					●	Ne	●
UM-38	Fresh				●ˢ				Ne	●
UM-39	Fresh				●ˢ			●ˢ	+	●
UM-40	Fresh		●ˢ			●ˢ			+	
UM-41	Fresh								+	
UM-42	Fresh				●ˢ	●ˢ			+	
UM-43	Fresh				●	●			+	
UM-44	Fresh		●ˢ					●ˢ	Ne	●
UM-45	Fresh		●			●			+	
UM-46	Fresh				●				Ne	●
UM-47	Fresh	●						●	+	
UM-48	Fresh			●ˢ				●ˢ	-	●
UM-49	Fresh	●						●	-	●
UM-50	Fresh		●ˢ						-	●
UM-51	Fresh				●ˢ			●ˢ	-	●
UM-52	Fresh				●ˢ			●ˢ	+	
UM-53	Fresh		●ˢ				●ˢ		+	
UM-54	Fresh		●ˢ			●ˢ			+	●
UM-55	Fresh								+	●
UM-56	Fresh		●ˢ					●ˢ	-	●
UM-57	Fresh				●ˢ			●ˢ	-	●
UM-58	Fresh		●					●	-	●
UM-59	Fresh		●ˢ					●ˢ	-	●
UM-60	Fresh		●ˢ						-	●
UM-61	Fresh		●ˢ			●ˢ			+	
UM-62	Fresh				●ˢ		●ˢ		+	
UM-63	Fresh		●ˢ			●ˢ			+	
UM-64	Fresh		●ˢ				●ˢ		+	
UM-65	Fresh		●ˢ					●ˢ	-	●
UM-66	Fresh		●					●	Ne	●
UM-67	Fresh		●ˢ					●ˢ	-	●
UM-68	Fresh				●ˢ		●ˢ		+	
UM-69	Fresh								+	
UM 70	Fresh				●			●	-	●

s; validated by sanger sequencing ****; intronic mutation***



Supplementary Fig 1. Copy number analysis of chromosome 1 and 8
A) SNP analysis indicates no loss of the entire chromosome 1 **B)** The absence of heterozygous variants in the B-allele frequency in the 1p arm of chromosome 1, indicates loss of 1p and normal 1q **C)** SNP analysis shows two copies of chromosome 8 **D)** Loss of the p-arm of chromosome 8 and allelic imbalance of the 8q arm.

Supplementary table 3. Chromosome status of chromosome 1p, 3 and 8q determined by Iontorrent SNP assay or other CNV analysis techniques

	Loss of chromosome 1p	Loss of chromosome 3	Gain of chromosome 8q
Iontorrent SNP assay	30% *(19/63)*	52% *(33/63)*	57% *(36/63)*
Other*	33% (20/61)	48% (29/60)	61% (37/61)

*** SNParray, MLPA or FISH analysis**