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Mutation frequencies of *GNAQ*, *GNA11*, *BAP1*, *SF3B1*, *EIF1AX* and *TERT* in uveal melanoma: detection of an activating mutation in the *TERT* gene promoter in a single case of uveal melanoma

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Background: Uveal melanoma is the most frequent primary tumour of the eye. It is molecularly clearly distinct from cutaneous melanoma and shows a different pattern of driver mutations. The influence of sunlight ultraviolet (UV) exposure on the aetiology of uveal melanoma is a matter of debate. The recent identification of driver mutations in the promoter of the telomerase reverse transcriptase (*TERT*) gene with UV-induced cytidine-to-thymidine transitions in cutaneous melanoma prompted us to investigate whether these mutations also occur in uveal melanoma.

Methods: We analysed 50 cases of uveal melanoma obtained from enucleation surgery for mutations in the genes *GNAQ*, *GNA11*, *BAP1*, *SF3B1*, *EIFAX1* and *TERT*, measured gene expression using microarrays and analysed gene copy numbers by SNP arrays.

Results: We detected a *TERT* mutation in only one case of a 57-year-old white male patient with clinical and histopathological features typical for uveal melanoma. The tumour showed mutations in *GNA11* and *EIF1AX* that are typical for uveal melanoma and absent from cutaneous melanoma. No mutations were detected in *GNAQ*, *BAP1* and *SF3B1* that are frequently mutated in uveal melanoma. Both copies of chromosome 3 were retained. Several tumours among which the one carrying the *TERT* promoter mutation showed elevated *TERT* expression. Consistent with previous reports, *GNAQ* is inversely associated with chromosome 3 monosomy and metastasis. *BAP1* mutations are significantly associated with chromosome 3 monosomy but not with relapse.

Conclusion: These data indicate that *TERT* mutations are rare in uveal melanoma. No conclusion can be drawn on their potential influence on tumour progression.

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Uveal melanoma is the most common primary tumour of the eye with an annual incidence of approximately two cases per million in southern European countries to eight cases in northern European countries (Virgili *et al*, 2007). Incidence increases with latitude in a highly significant manner (Virgili *et al*, 2007). Whether this association can be attributed to the exposure to sunlight of variable intensity or not, remains a matter of discussion (Gallagher *et al*, 1985; Pane and Hirst, 2000; Guenel *et al*, 2001; Singh *et al*, 2004). Uveal melanoma shows a mutation pattern that is clearly distinct from cutaneous (for recent reviews, see Coupland *et al*, 2013; Harbour, 2013; Zeschnigk and Lohmann, 2013), mucosal (Furney *et al*, 2013) and conjunctival melanomas (Griewank *et al*, 2013c). The mutations typically encountered in cutaneous and conjunctival melanomas, BRAF and NRAS, are rare in uveal melanomas that are characterised by mutations of the G-proteins GNAQ and GNA11 occurring in mutual exclusive manner in ~85% of the cases (Zeschnigk and Lohmann, 2013). The mutation pattern observed by exome sequencing in cutaneous melanoma is clearly consistent with an aetiological role of sunlight exposure (Hodis *et al*, 2012). Cytidine-to-thymidine transition frequency has not been addressed by the two exome sequencing studies of uveal melanoma (Harbour *et al*, 2013; Martin *et al*, 2013) but the complete lack of overlap among the driver mutations in the two pathologies is consistent with a different aetiology.

Recently, three mutations in the promoter of the telomerase reverse transcriptase (*TERT*) needed for telomere maintenance in cancer cells, close to the transcriptional start site, have been described for sporadic (Huang *et al*, 2013) and familial (Horn *et al*, 2013) forms of cutaneous melanoma. The familial cases all derived from one family and showed a mutation different from those detected in sporadic melanomas and melanoma cell lines (Huang *et al*, 2013). Two mutations were identified in sporadic cases where they occurred in a mutually exclusive manner in over 70% of cases. The same mutations were also present, though less frequently, in other tumours and tumour-derived cell lines. The mutations consisted in cytidine-to-thymidine transitions at a dipyrimidine motif consistent with ultraviolet (UV) light-induced damage. All three mutations created novel binding sites for the transcription factor E-twenty-six (ETS) in the *TERT* promoter within 100 bp upstream of the transcription start site (TSS) in sporadic cases and closer to the TTS (-57 bp) in the familial cases. Reporter assays showed the functionality of the new binding sites observed in sporadic cases (Huang *et al*, 2013). The mutation is highly penetrant in the melanoma family analysed and it is linked to early onset (mean 34 years) and short survival (mean 3.5 years in 8 patients who died from the disease of a total of 11 cases). It is therefore probable that the mutation strongly contributes to the development of aggressively growing cutaneous melanoma.

Griewank *et al* (2013a) have addressed whether the same mutations are present also in ocular melanomas reporting an incidence of 32% in conjunctival melanomas and the absence from uveal (ciliary body or choroid) melanomas ($n = 47$).

In the present study, we describe the analysis of *TERT* promoter mutations in a series of 50 uveal melanomas. A mutation was detected in a single case that also carried mutations in *GNA11* and *EIF1AX*, typical for this disease and absent from conjunctival and cutaneous melanomas. The tumour showed disomy of chromosome 3 and had wild-type sequences of *GNAQ*, *BAP1* and *SF3B1* which frequently show mutations in uveal melanoma.

MATERIALS AND METHODS

Patients. Tissue samples were obtained from 52 primary uveal melanomas after enucleation surgery upon approval of the institutional bioethics board and informed written consent of

the patients. Clinical, pathological and molecular characteristics and follow-up data are reported in Supplementary Table 1.

DNA/RNA extraction, array comparative genome hybridisation, MPLA, gene expression profiling and Sanger sequencing. DNA was extracted from macrodissected tumour material conserved in RNAlater (Ambion, Monza, Italy) or from archival formalin-fixed, paraffin-embedded (FFPE) blocks using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). For FFPE samples, two 10- μm -thick sections were cut from each paraffin block and subjected to de-paraffinisation using scalar concentrations (100% to 40%) of Histoclear (Sigma Aldrich, Milan, Italy), followed by re-hydration and overnight digestion with proteinase K (100 $\mu\text{g ml}^{-1}$, Sigma Aldrich) at 37 °C. DNA concentration and quality were checked in the Nanodrop ND-1000 spectrophotometer and integrity of DNA was tested by agarose gel electrophoresis. Processing of genomic DNA was performed using the GeneChip Mapping 250 K Assay Kit (Affymetrix, Santa Clara, CA, USA) as recently described (Amaro *et al*, 2013). Briefly, 250 ng of DNA sample were digested with the restriction enzyme NspI. Adapters were ligated using T4 DNA Ligase. Whole genome amplification was performed on a GeneAmp PCR System 9700 (Applied Biosystems–Life Technologies Corporation, Monza, Italy). Ninety micrograms of amplified and normalised PCR product were fragmented and labelled. Hybridisation, washing, staining and scanning of SNP arrays were performed on the Affymetrix fluidic station. Quality of the samples was assessed on agarose gels before the hybridisation step. Affymetrix Genotyping Console (GTC4.1.2) was used to perform genotype call and quality control assessments. Copy number analysis was performed using CNAG3.0 (Yamamoto *et al*, 2007).

Multiplex ligation-dependent probe amplification (MLPA) was performed according to the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands). In short, 5 μl of genomic DNA diluted in TE at a concentration of 20 ng ml^{-1} were denatured at 98 °C for 5 min, cooled to 25 °C and 3 μl of a 1:1 mixture of MLPA buffer and SALSA P027.C1 probe-mix was added. After hybridisation for 16 h at 60 °C, 32 μl ligation mix were added, and the reaction was incubated for 15 min at 54 °C followed by 5 min at 98 °C. Subsequently, 10 μl of the SALSA PCR-mix (FAM label) were added to 40 μl of ligation product and this was amplified by PCR in 35 cycles (30 s, 95 °C; 30 s, 60 °C; 60 s, 72 °C). PCR products were quantified using the ABI 3130XL (Applied Biosystems–Life Technologies Corporation, Monza, Italy) and Coffalyser.net software (MRC-Holland). Raw data were expressed as peak heights, as a measure of peak intensity, for each of the 50 probes (38 test probes and 12 control probes). For each probe, the relative ratio was calculated using population normalisation and using the internal control probe normalisation. The MLPA data were considered reliable if six or more control probes were within the normal range. Loss was identified as MLPA relative ratio <0.70 and gain as MLPA relative ratio >1.3.

For RNA extraction, tumour samples were homogenised in the tissue lyserMixer Mill (Qiagen) in total RNA extraction lysis buffer using RNeasy (Qiagen). RNA quality was assessed in the BioAnalyser (Agilent, St Clara, CA, USA). Gene expression profiling was performed using Affymetrix HGU133plus2 arrays following standard procedures as described (Gangemi *et al*, 2012). Assignment to prognostic gene expression classes was performed by nearest neighbour classification using the gene expression values for the discriminator genes described (Onken *et al*, 2004).

PCRs were carried out using 25 ng of genomic DNA in a 25 μl reaction mix including 10 \times Platinum PCR Supermix, 1.5 mM MgCl₂, 200 μM dNTPs, 1 μM primers and 0.5U Taq Platinum (Invitrogen–Life Technologies Corporation, Monza, Italy) on a Veriti 96 wells Thermal Cycler (Applied Biosystems–Life Technologies Corporation). Thermal cycling conditions were as

follows: 94 °C for 4 min, followed by 25 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 7 min. PCR conditions were slightly modified for *EIF1AX* gene (2 mM MgCl₂ were used for exon 1 and the annealing temperature was 54 °C for exon 1 and 64 °C for exon 2).

Telomerase reverse transcriptase promoter region oligonucleotide primers were synthesised according to the Eukaryotic Promoter Database genomic reference sequence of *TERT* and designed to amplify a portion of the *TERT* core promoter (−144 to +43). The amplicon of 187 bp includes the two major recurrent mutations at coordinates chr 5:1295228 and chr 5:1295250. PCR were performed by using 100–200 ng of genomic DNA in a 50 μl reaction mix with 400 μM dNTPs (Fermentas-Thermo Scientific Life Science, Milan, Italy), 0.75 μM primers, 2 mM MgCl₂, 1X Buffer and 2.5 U AmpliTaq Gold 360 DNA Polymerase (Applied Biosystems-Life Technologies Corporation) on a Veriti 96 wells Thermal Cycler (Applied Biosystems, Life Technologies Corporation). Thermal cycling conditions were as follows: 95 °C for 7 min, followed by 42 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min.

All PCR primers (see Supplementary Table 2) except for *TERT* were designed with a universal sequence at 5'-end (universal forward primer 5'-GTTGTAAAACGACGGCCAGT-3' and M13 (−48) reverse primer 5'-GTGTGAAATTGTTATCCGCT-3') to perform single-pass sequencing. Additional primers were synthesised for FFPE samples that did not yield any amplicon using the first set of primers. Mutational screening was carried out by direct

sequencing of fragments obtained by PCR using an ABI3130xl and ABI3730 Genetic Analyzer (Applied Biosystems–Life Technologies Corporation). Sequencing data were analysed using SeqScape v2.5 software (Applied Biosystems–Life Technologies Corporation) and MacVector V11 software (MacVector Inc., Cary, NC, USA).

Transcription factor binding analysis was performed using TFSEARCH based on the TRANSFAC database (Heinemeyer *et al*, 1998).

RESULTS

From the available 52 tumours, sufficient amounts of DNA could be obtained from 51 tumours and 50 of which could be successfully sequenced for *TERT*. One (MU076) of these 50 cases showed a heterozygous mutation in the promoter of *TERT*, a C to T transition on chromosome 5 in position 1295228 (Figure 1A) that increases the likelihood of the sequence to bind ETS transcription factors from 78.4 (wild type) to 86.3 (mutation) as analysed by TFSEARCH and has been shown to drive *TERT* promoter activity (Huang *et al*, 2013). The same tumour showed a heterozygous *GNA11* mutation in exon 5 (Figure 1B) and wild-type sequences for *BAP1* and *SF3B1* (data not shown). *EIF1AX* exon 2 showed a mutated sequence in addition to the wild-type allele (Figure 1C).

The mutation spectrum of genes that are frequently mutated in uveal melanoma is compatible with the frequencies observed in

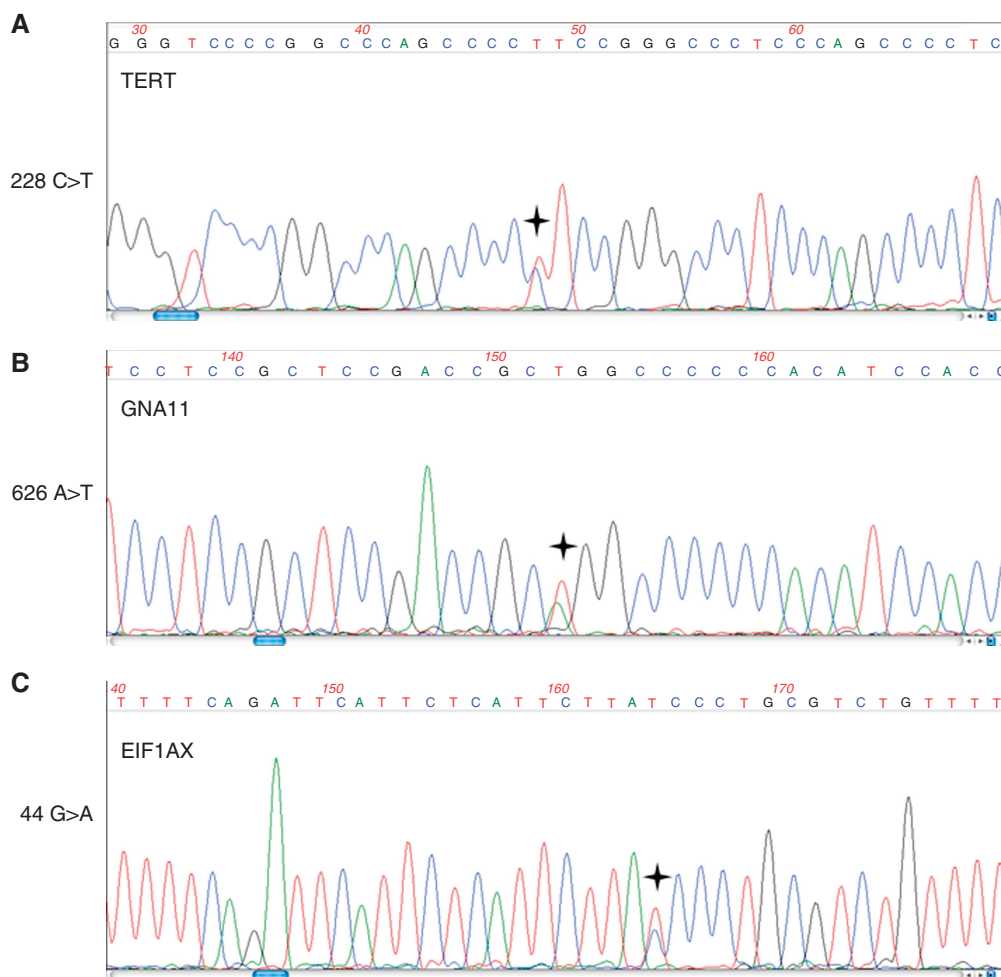


Figure 1. Mutation analysis by Sanger sequencing. The genes *TERT* (A), *GNA11* (B) and *EIF1AX* (C) were found to be mutated as indicated on the left of each diagram by Sanger sequencing using the primers enlisted in Supplementary Table 2. The asterisk indicates the mutated position. No mutations have been detected in *GNAQ*, *BAP1* and *SF3B1* (see text).

previous reports (Martin *et al*, 2013; Griewank *et al*, 2013b). *EIF1AX* mutations exclusively occurred in disomic, non-metastatic cases. One of the three *SF3B1* mutations detected occurred in a metastatic case with uniparental disomy of chromosome 3. *GNAQ* and *GNA11* mutations accounted for ~84% of the cases. *GNAQ* was more frequently observed in disomic cases, *GNA11* more frequently in monosomic cases. *BAP1* was significantly more frequent in monosomic cases, however, due to several monosomic, progression-free cases with long follow-up, *BAP1* was not significantly associated with metastasis. *GNAQ* was inversely associated with metastasis (see Table 1).

Cytogenetic analyses divide uveal melanomas into two distinct patterns: the first, detected in ~50% of uveal melanomas,

characterised by loss of chromosome 3, often associated with chromosome 8 gain and strongly linked to metastatic disease. The second pattern accounts for 25% of uveal melanomas and is characterised by chromosome 6p gain, often associated with 6p loss but rarely with chromosome 3 loss (Hoglund *et al*, 2004). Copy number alteration analysis of the uveal melanoma carrying the *TERT* promoter mutation using Affymetrix 250K SNP arrays revealed disomy of chromosome 3 and copy number gain of the short arm of chromosome 6. No extended regions of homozygosity compatible with uniparental disomy of chromosome 3 could be identified (Figure 2).

The tumour occurred in the posterior chamber of the right eye of a 57-year-old male patient of European ancestry, had a depth of 11.97 mm and a width of 12.62 mm, showed the characteristic mushroom shape, epitheloid histology and a low mitotic index (Figure 3). The tumour was removed by enucleation of the eye. The patient is free of disease 31 months after diagnosis.

The application of the prognostic gene set described by Onken *et al* (2004) to this case classifies it as class 1 tumour associated with low risk. The expression of *TERT* as revealed by microarray gene expression data shows that the tumour carrying the mutation, as well as several other tumours with wild-type *TERT* promoters, has elevated levels of *TERT* expression as compared with the mean of *TERT* expression in the whole cohort (Figure 4). The six tumours with the highest *TERT* expression levels did not metastasise (mean follow-up 33.3 months, range 16–64 months). Four of these tumours have two chromosomes 3, this information is not

Table 1. Mutation frequencies

Mutation	Total (%)	Disomic cases (%)	Monosomic cases (%)
<i>GNAQ</i> (n = 45)	19 (42.2)	14 (73.7)	5 (26.3)
<i>GNA11</i> (n = 46)	15 (32.6)	6 (40.0)	9 (60.0)
<i>BAP1</i> (n = 38)	12 (31.5)	0 (0.0)	12 (100.0)
<i>SF3B1</i> (n = 31)	3 (9.7)	3 (100.0)	0 (0.0)
<i>EIF1AX</i> (n = 37)	7 (18.9)	7 (100.0)	0 (0.0)
<i>TERT</i> (n = 50)	1 (2.0)	1 (100.0)	0 (0.0)

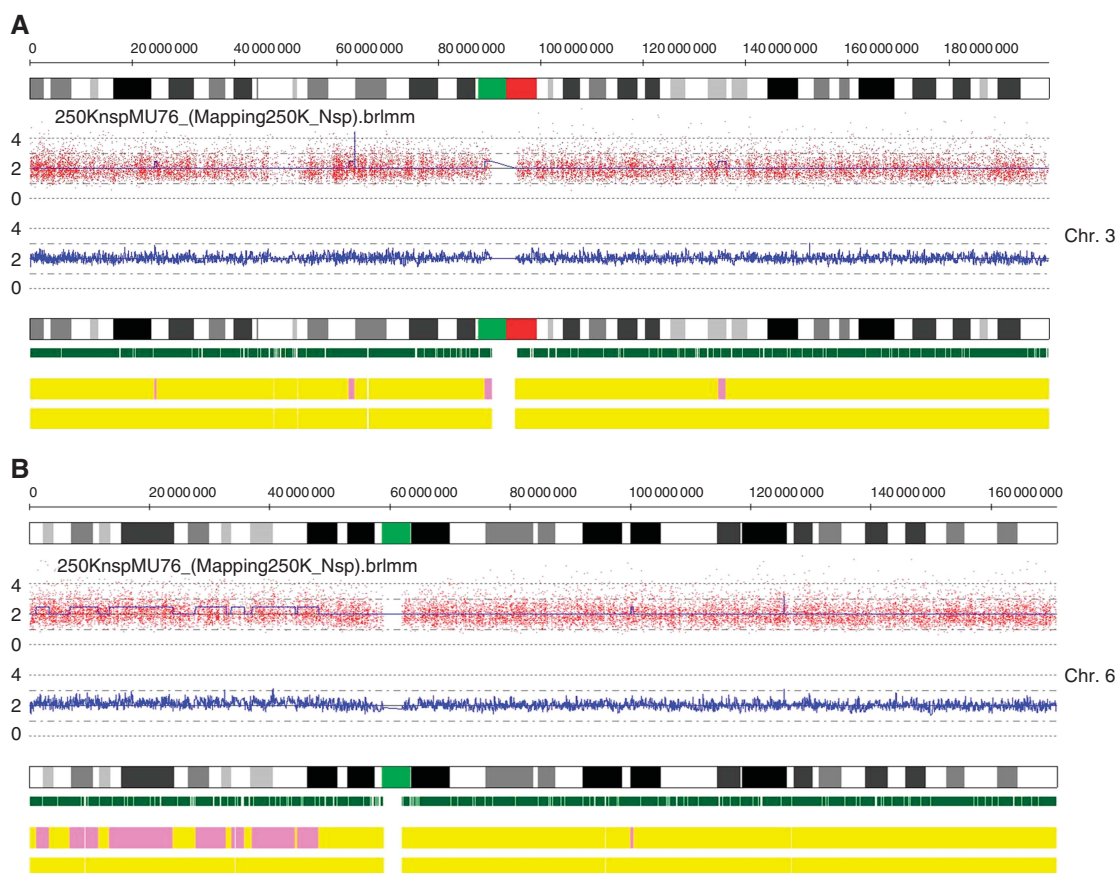


Figure 2. Copy number alteration of chromosomes 3 and 6. DNA extracted from the tumour sample was analysed by arrayCGH on Affymetrix 250 K SNP arrays. Chromosome 3 (A) is present with two copies that, based on SNP analysis, are heterozygous. Short amplified stretches but no deletions are observed. Chromosome 6q (B) shows extended stretches with presumably three copies. On top of each diagram the chromosome bands are represented. The plot below shows the actual intensities; the blue plot corresponds to the copy number call applying a five SNP window. The green vertical bars show the positions of the single probe sets of the array, the upper yellow bar indicates disomy (= yellow) or gain (= pink) and the lower bar indicates probability of loss of heterozygosity (<30%= yellow).

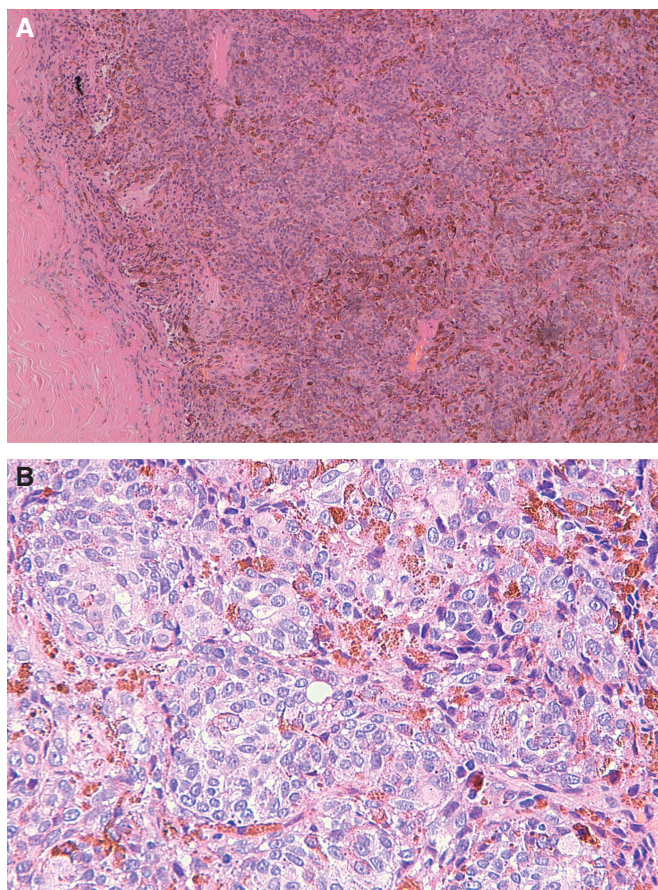


Figure 3. Clinical and histopathological features of the tumour carrying the *TERT* promoter mutation. (A) Solid growth pattern of atypical epithelioid melanocytes. Irregular distribution of melanin. (B) Confluent nests of atypical epithelioid melanocytes. Very rare mitoses.

available for the other two. However, despite this trend, high *TERT* expression levels are not significantly associated with disease-free survival ($P=0.338$) or other clinical, pathological or molecular features of the tumours (Figure 4; supplementary table 1).

DISCUSSION

The analysis of the recently identified uveal melanoma driver mutations in the genes *GNAQ*, *GNA11*, *BAP1*, *SF3B1* and *EIF1AX* generally confirms the observed mutation frequencies and the prevalence of *SF3B1* and *EIF1AX* mutations in disomic, non-metastatic cases. Also the mutual exclusive mutations of *GNAQ* and *GNA11* and the association of the latter with a more aggressive tumour phenotype could be confirmed. Mutations of the tumour suppressor gene *BAP1* are associated with monosomy of chromosome 3 yet in our cohort, there are several cases without metastases despite monosomy of chromosome 3 and *BAP1* mutation. Although this is probably due to chance it underlines the limited prognostic value of mutation analyses.

In addition, we show here a case of uveal melanoma that carries one of the two mutations in the promoter region of *TERT* that have been described for sporadic cutaneous melanoma that closely resembles cutaneous melanoma with which it shares *BRAF* and *NRAS* mutations (Griewank *et al*, 2013a) (and references therein). In fact, *TERT* mutations in conjunctival melanomas also occurred in concomitance with *BRAF* and *NRAS* mutations (Griewank *et al*, 2013a). However, Griewank *et al* (2013a) did not detect *TERT*

promoter mutations in 47 uveal melanomas. Hence, the *TERT* mutation identified here has an estimated frequency of 1 in 97 (~1%) in uveal melanoma. The tumour carrying this mutation can clearly be identified as a uveal melanoma as it was located in the posterior chamber of the eye, showed the typical epithelioid morphology and carries a mutation in the *GNA11* gene. *GNA11* mutations occur in 33.2% of uveal melanomas and in 6.5% of blue nevi but are absent from other nevi ($n=105$) and extraocular melanomas ($n=273$) (Van Raamsdonk *et al*, 2010; see Supplementary Table 2 for mutation frequencies of *GNAQ*, *GNA11*, *BAP1*, *SF3B1* and *EIF1AX* as obtained from the Catalogue of somatic mutations in Cancer (COSMIC) database; <http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/>). We can therefore be certain of the uveal origin of the tumour that carries the *TERT* mutation.

The mutation in *EIF1AX* has also been shown to be frequent in uveal melanoma. *EIF1AX* is located on the X chromosome and, being the patient of male sex (confirmed by analysis of X chromosome number by Devyser QF-PCR and several Y-chromosome markers; data not shown), the appearance of two alleles can most likely be attributed to the presence of the mutation in only a subclone of the tumour. The latter appears more likely as the presence of non-tumoral components is not evident for *GNA11* and *TERT* mutations. We can exclude contamination with the Y-chromosome paralogue, *EIF1AY*, as the primers used are identical to those described by Martin *et al* (2013) and do not amplify this gene. The occurrence of an *EIF1AX* mutation in a disomic case with a *GNA11* mutation but without *BAP1* and *SF3B1* mutations is consistent with the original report of this mutation (Martin *et al*, 2013).

Huang *et al* (2013) have shown that the *TERT* promoter mutation determines increased promoter activity in a luciferase reporter system. We show here for the first time that the mutation in the promoter is actually associated with increased expression of the gene in the tumour. However, several wild-type tumours also show increased *TERT* expression indicating that there are other means to increase the expression of the catalytic subunit of human telomerase. Telomere maintenance might therefore be important in at least a small subgroup of uveal melanomas. Increased *TERT* expression is expected to facilitate immortalisation of somatic cells as an essential step in neoplastic transformation as the replicative potential of normal cells is limited by telomere shrinkage (Bodnar *et al*, 1998). Telomerase reverse transcriptase promoter mutations have been observed in a variety of tumours and it has been speculated that these mutations could be more frequent in tumours derived from tissues with low self-renewal where tumour development might be hindered by the inherently lower telomerase activity (Killela *et al*, 2013). Telomere shrinkage can also be overcome in cancer cells by mutations in the genes alpha thalassemia/mental retardation syndrome X-linked (*ATRX*) or death-domain-associated protein (*DAXX*) leading to recombination-mediated telomere maintenance (Heaphy *et al*, 2011). No such mutations have been found by exome sequencing of uveal melanomas (Griewank *et al*, 2013a).

The case carrying this mutation shows disomy of chromosome 3 and wild-type status of the tumour suppressor gene *BAP1* that is frequently mutated in tumours that undergo metastasis (Harbour *et al*, 2010) and that show a chromosome 3 monosomy-associated gene expression profile (Onken *et al*, 2004, 2012). The epithelioid morphology of the tumour cells is associated with increased risk of metastasis (Singh *et al*, 2001). Given the strong prognostic effect of chromosome 3 monosomy, this tumour would be considered as at low risk of metastasis despite epithelioid morphology and tumour dimensions. The patient whose uveal melanoma carried the *TERT* mutation is free of disease at 31 months after diagnosis but an effect of the mutation in the *TERT* promoter on the metastasis risk

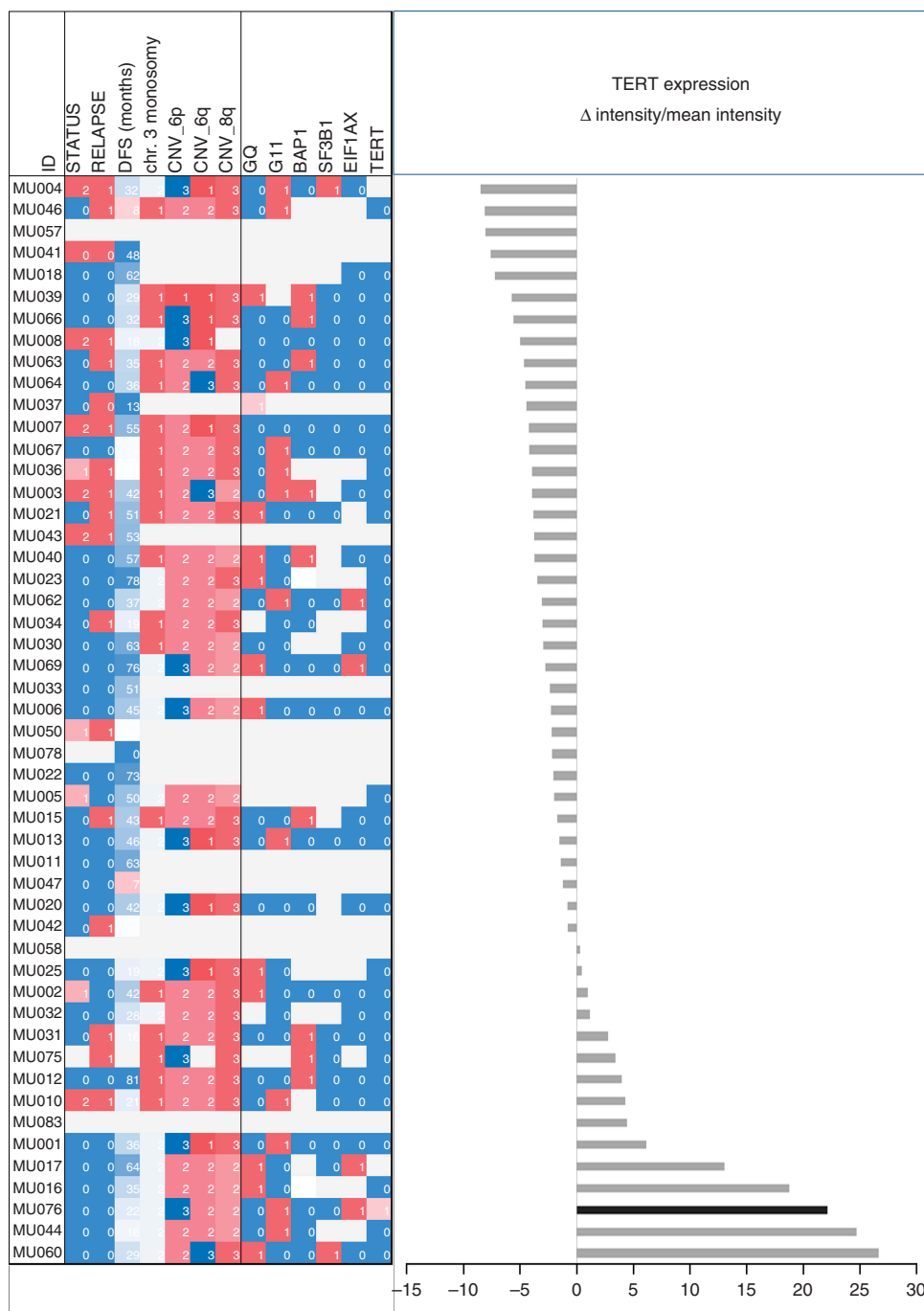


Figure 4. TERT gene expression and tumour characteristics. The differences between microarray intensity values measured for the single cases and the mean of the whole cohort are plotted (right panel). The case carrying the TERT promoter mutation is indicated in black. Clinical, histopathological and molecular characteristics are indicated (left panel): status (0 = alive, 1 = death of other causes, 2 = death of uveal melanoma metastasis), relapse (0 = relapse free, 1 = relapse), CNVs (1 = monosomic or losses, 2 = disomic, 3 = trisomic or gains), mutations (0 = wild type, 1 = mutated). For details see Supplementary Table 1.

cannot be definitely excluded. No information on the association of TERT promoter mutations with prognosis is available, not even for cutaneous melanoma as this has not been addressed by the original studies (Horn *et al*, 2013; Huang *et al*, 2013). The mutation detected in the melanoma family showed high penetrance and occurred in aggressive melanomas with early onset and short survival after diagnosis even in some cases of the last generation where present day management of the disease can be assumed (Horn *et al*, 2013). Yet this specific mutation is different from those detected in sporadic cases and in the present case of uveal

melanoma and the precise location within the promoter and/or the location with respect to other promoter elements could affect the extent of promoter activation. On the other hand, several cell lines derived from metastatic melanomas showed the mutation identified in sporadic cases independently of known driver mutations consistent with driver function for the TERT promoter mutation (Huang *et al*, 2013). It is therefore possible that the mutation influences the overall risk of metastasis.

If so, this mutation could be associated with the rare cases of uveal melanoma with disomy and BAP1 wild type that develop

metastases and that, though rare, determine pitfalls in prognostic testing (Zeschnigk and Lohmann, 2013). *GNAQ* and *GNA11* mutations are not sufficient for the generation of a metastatic phenotype of uveal melanoma (Van Raamsdonk *et al*, 2009, 2010). In most cases, the metastatic phenotype is acquired only after loss of one copy of chromosome 3 and *BAP1* mutation (Coupland *et al*, 2013; Harbour, 2013; Zeschnigk and Lohmann, 2013). The association of the *GNA11* mutation with another driver mutation in *TERT* might drive a more aggressive phenotype independently of chromosome 3 monosomy and *BAP1* mutation. Analysis of *TERT* should therefore be included in the assessment of uveal melanomas in order to reveal its real frequency and to investigate into its association with metastasis.

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