POT1 and Damage Response Malfunction Trigger Acquisition of Somatic Activating Mutations in the VEGF Pathway in Cardiac Angiosarcomas

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Background—Mutations in the POT1 gene explain abnormally long telomeres and multiple tumors including cardiac angiosarcomas (CAS). However, the link between long telomeres and tumorigenesis is poorly understood.

Methods and Results—Here, we have studied the somatic landscape of 3 different angiosarcoma patients with mutations in the POT1 gene to further investigate this tumorigenesis process. In addition, the genetic landscape of 7 CAS patients without mutations in the POT1 gene has been studied. Patients with CAS and nonfunctional POT1 did not repress ATR (ataxia telangiectasia RAD3-related)–dependent DNA damage signaling and showed a constitutive increase of cell cycle arrest and somatic activating mutations in the VEGF (vascular endothelial growth factor)/angiogenesis pathway (KDR gene). The same observation was made in POT1 mutation carriers with tumors different from CAS and also in CAS patients without mutations in the POT1 gene but with mutations in other genes involved in DNA damage signaling.

Conclusions—Inhibition of POT1 function and damage-response malfunction activated DNA damage signaling and increased cell cycle arrest as well as interfered with apoptosis, which would permit acquisition of somatic mutations in the VEGF/angiogenesis pathway that drives tumor formation. Therapies based on the inhibition of damage signaling in asymptomatic carriers may diminish defects on cell cycle arrest and thus prevent the apoptosis deregulation that leads to the acquisition of driver mutations. (J Am Heart Assoc. 2019;8:e012875. DOI: 10.1161/JAHA.119.012875.)

Key Words: cardiac angiosarcoma • cell cycle arrest • damage response • POT1 • VEGF/angiogenesis pathway

The Li-Fraumeni syndrome is an autosomal dominant syndrome representing a genetic predisposition to a wide spectrum of tumors and is typically linked to mutations of the TP53 tumor suppressor gene.1 Li-Fraumeni-like families have a similar clinical presentation, but Li-Fraumeni-like syndrome is less frequently associated with mutations in the TP53 gene. Recently, we studied different Li-Fraumeni-like families with multiple tumors including various cases of cardiac angiosarcoma (CAS), which is the most common and most aggressive type of primary malignant neoplasm of the heart in adults.2 Patients affected with CAS are generally diagnosed at advanced stages with very poor prognosis and...
Clinical Perspective

What Is New?

- In this study we describe how mutations in the \( \text{POT1} \) gene, which explain long telomeres, correlate with cell cycle arrest increase in angiosarcoma patients.
- The same increase was observed in other cardiac angiosarcoma patients even without mutations in the \( \text{POT1} \) gene but in the damaged response signaling.
- This malfunction would bypass the apoptosis mechanism and would trigger the acquisition of somatic activating mutations in the angiogenesis pathway.

What Are the Clinical Implications?

- Our results suggest that the use of angiogenesis inhibitors might regulate the tumor progression; however, targeting ATM/ATR (ataxia telangiectasia mutated/RAD3-related) activity would rescue the cell cycle control and would prevent the acquisition of somatic driver mutations in patients affected with angiosarcoma tumors and asymptomatic patients carrying \( \text{POT1} \) mutations.

short survival rates (5-year survival rate of 14%). The genetic landscape that determines the tumorigenic process of angiosarcomas (AS) is poorly understood and not well established.4,5 Previous studies by our group uncovered a deleterious missense mutation in the \( \text{POT1} \) gene (c.349C>T [p.Arg117Cys], pathogenic, Li-Fraumeni-like syndrome/CAS, autosomal dominant)6 causing AS in 4 families (3 in cardiac tissue and 1 in breast).6 Germline mutations in the \( \text{POT1} \) gene have also been related with the development of other familial cancer types.7-12 \( \text{POT1} \) is a component of the so-called shelterin complex, which is involved in telomere elongation in germline and stem cells (Figure 1A).13 In normal conditions the shelterin complex protects telomere cap ends in somatic cells by preventing access of the telomerase to chromosome ends.14 The shelterin complex also masks single-stranded telomeres from the DNA damage response, thereby preventing the activation of ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia RAD3-related) to avoid cell cycle arrest through \( \text{POT1} \) and TPP1 (Figure 1B).15 \( \text{TPP1} \) (which is also called \( \text{ACD} \) gene) anchors the telomere by \( \text{POT1} \) and TRF1 (telomeric repeat binding factor 1) proteins. When telomeres are critically short, the shelterin complex does not prevent activation of the ATM and ATR response, which can drive the cell to senescence and apoptosis (Figure 1C).

Our previous studies demonstrated that cardiac angiosarcoma patients carrying the \( \text{POT1} \) p.Arg117Cys mutation showed abnormally long telomeres due to the lack of repression of telomerase, which led to increased fragility and damage.6 Other described \( \text{POT1} \) mutations associated with risk of developing familial glioma and familial melanoma tumors also led to abnormally long and fragile telomeres.7 However, the link between telomere instability and tumorigenesis is not well understood. In addition, studies of the biological pathways involved in the progression of angiosarcomas are very scarce. Recently, next-generation sequencing studies uncovered somatic alterations in the VEGF/PLCG1 pathway in cardiac angiosarcoma16,17 and driver mutations in the PI3K/AKT/mTOR and MEK pathways in angiosarcomas other than cardiac,18-21 but these studies did not distinguish between constitutive and somatic mutations and did not clarify the genetics and underlying mechanisms.

In order to understand how telomere instability links the angiosarcoma process, in the present work we studied somatic events in different angiosarcoma patients carrying the \( \text{POT1} \) p.Arg117Cys mutation described in Calvete et al6; 2 patients (F1 and F3) affected with CAS and 1 patient (F2) with 2 tumors, a breast AS and a papillary thyroid tumor. In addition, we studied the genetic and molecular somatic landscape that drives tumor progression in 7 patients with sporadic CAS tumors (NT1-NT3 and T1 to T4) who did not carry mutations in the \( \text{POT1} \) gene.

Methods

The data, methods used in the analysis, and materials used to conduct the research are available to any researcher for purposes of reproducing the results or replicating the procedure.

Ethics Statement

Institutional Review Board approval was obtained; the ethics committee of the CNIO, the Institute of Health Carlos III, and SaludMadrid (Autonomous Community of Madrid) approved this study (CS9679). Informed consent was received from participants before inclusion in the study.

Patients

Formalin-fixed paraffin-embedded (FFPE) tissue samples from 10 patients were selected. The 3 familial angiosarcoma individuals carrying the \( \text{POT1} \) p.Arg117Cys mutation were selected from Calvete et al.6 Seven FFPE tissue samples from individuals affected with sporadic CAS not carrying mutations in the \( \text{POT1} \) gene were also selected: 3 FFPE tissue sections contained normal (N) and tumor (T) tissue (NT1, NT2 and NT3), and the other 4 FFPE sections contained only tumor tissue (T1 to T4).
Genomic DNA was extracted from FFPE tissue samples and from fixed tissue slides (microdissection) using the DNeasy Blood & Tissue Kit (Cat No. 69504, Qiagen, Hilden, Germany) following the manufacturer’s instructions. Histology of hematoxylin and eosin–stained sections of the tissues was assessed by a pathologist (M.M.).

Immunohistochemistry

FFPE blocks were cut into 5-μm-thick sections for immunohistochemistry (IHC) studies. The cell cycle in normal tissue was tested with anti-p21 (WAF1) from Merck (Darmstadt, Germany; Ref MABE325), anti-p27 (57/Kip1) from BD Biosciences (Franklin Lakes, NJ; Ref 610242), and anti–phospho-histone γH2AX (Ser139) from Millipore (Burlington, MA; Ref 05-636) antibodies. Activation of the VEGF-angiogenesis pathway was assessed with two different antibodies against phosphorylated (activated) proteins: anti–phospho-p44/42-MAPK (ERK1/2) and anti–phospho-S6 ribosomal protein (Ser235/236) from Cell Signaling Technology (Danvers, MA; Refs 9101 and 2211, respectively). Both the absence of staining and excess nonspecific staining were considered as negative staining. Staining was considered separately in normal (N) and tumor (T) tissues. IHC controls were performed by using normal tissue from biopsies of healthy individuals.

Samples

Genomic DNA was extracted from FFPE tissue samples and from fixed tissue slides (microdissection) using the DNeasy Blood & Tissue Kit (Cat No. 69504, Qiagen, Hilden, Germany) following the manufacturer’s instructions. Histology of hematoxylin and eosin–stained sections of the tissues was assessed by a pathologist (M.M.).

Whole Exome Sequencing and Bioinformatics Pipelines

Exomes from selected tissues were captured and enriched using the SureSelect Human All Exon Kit (78 Mb) (Agilent Technologies, Santa Clara, CA). Enriched samples were paired-end sequenced on an Illumina Genome Analyzer II sequencing platform using 2 lanes per sample and generating 101 base-pair long reads. FASTQ files of short reads were aligned using the BWA algorithm to the GRCh37/hg19 reference genome. 

GATK-based variant calling was performed for aligned reads considering DP (Read Depth) values of >30 and Quality-by-Depth scores for a variant confidence of >1.00. A total of 92.28% (ranging from 91.70% to 92.16%) of the variants that were well aligned and annotated passed the quality and coverage filters. Strict filtering for only well-defined variants by quality controls, and those not included in repeat regions were included to prevent false positives. Tumor
variants (<10% alternative variant allele frequency) and those with low coverage (<6x), were discarded.

Only quality-filtered variants affecting coding sequences of canonical transcripts (nonsynonymous, essential splice site, frame shift or gain/loss of stops) were taken into account. Variant type annotation, population statistics, disease-specific sequence databases, and in silico predictive algorithms were according to AMCG standards and guidelines. Variants with a minor allele frequency of <0.01 and <0.05 (dbSNP130, HapMap, or 1000 Genomes) were considered for stringent and relaxed filtering, respectively. Their potential damaging effect was assessed using the VEP script software package (including Sift, Polyphen and Condel damage predictors). Stringent filtering only considered the variants annotated as pathogenic by all damage predictors. Whole-exome sequencing data have been deposited in the ArrayExpress public database under accession number E-MTAB-7999 (available at https://www.ebi.ac.uk/fg/annotare/).

Variants found in DNA blood samples and variants found in common between paired normal/tumor tissues were considered as constitutional. Variants found only in tumor tissue were considered somatic variants. Constitutional variants were validated in DNA from blood and normal tissue samples, and somatic variants were validated in DNA from tissue samples (tumor) by Sanger sequencing.

Pathway Enrichment Analyses

Two different software packages were used for independent assessments of the gene set analyses. Data were analyzed with Qiagen’s Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, CA www.qiagen.com/ingenuity) and ConsensusPathDB (available at http://cpdb.molgen.mpg.de/). Overrepresentation analysis of the gene set list was performed with a minimum overlap of 4 genes with the pathway database set size and a P-value cutoff of 0.001. Induced network module analysis without intermediate nodes and considering high-confidence binary protein and genetic/gene regulatory interactions were also evaluated.

Trusight Tumor 170 Panel and IBM Watson Study

Resequencing of tumor DNA from FFPE tissue samples was performed with TruSight Tumor 170 from Illumina (San Diego, CA), a novel sequencing platform that predicts the most probable variant causing the pathology and provides suggestions for translating the data to the clinic. The sequencing of the hybrid capture was run on the HiSeq 2500 System. The captured gene content in the TruSight Tumor 170 Assay, data sheet, and specifications are available at https://www.illumina.com. Low variant quality of <20 and low depth (<100 for variant calls and <250 for reference call filters) were considered. Variants were supported with >7 reads and filtered by frequency (minor allele frequency <0.05). The copy number variation call was calculated for the fold-change results for each gene. Variants were classified and analyzed later by the IBM Watson for Genomics platform, which searches electronic medical databases to find information that may be relevant to a particular genomic sequence (available at https://www.ibm.com/watson/).

Results

Angiosarcoma Patients Carrying the POT1 p.Arg117Cys Mutation

Constitutional Effect in Normal Tissue of POT1 p.Arg117Cys Mutation Carriers

In normal conditions the POT1 protein represses downstream activation of the DNA damage response at telomeres in somatic cells (Figure 1B). The POT1 p.Arg117Cys protein shows a reduced capacity to bind telomeres and TPP1 and may affect the regulation of the damage response. Damage response activation leads to cell cycle arrest, replicative senescence, and apoptosis (Figure 1C). In order to decipher the putative effect of POT1 malfunction, patient tissues were stained with anti-P-γH2AX (DNA damage marker) and anti-p21 and anti-p27 antibodies (inhibitors of CDK1/2 to arrest the cell cycle). IHC studies were carried out in normal (N) tissues of patients from the families carrying the constitutional POT1 p.Arg117Cys mutation studied in Calvete et al: 2 patients (F1 and F3) affected with CAS and 1 patient (F2) with 1 breast AS and 1 papillary thyroid tumor.

Increased IHC staining with anti-p21, -p27, and -P-γH2AX antibodies was observed in N tissues of all studied patients in comparison with the corresponding N tissues from healthy non–mutation carriers (Table 1 and Figure 2A). Therefore, the reduced binding to telomeres and to TPP1 by the POT1 p.Arg117Cys protein correlates with activation of damage response signaling mediated by p21 and p27 (Figure 1D).

Somatic Events in POT1 p.Arg117Cys Mutation Carriers

To evaluate possible somatic events in affected individuals carrying the POT1 p.Arg117Cys mutation that might lead to the formation of AS, the exomes of normal (N) and tumor (T) tissues of the F1 and F2 individuals were sequenced. Variants found in T tissue but not in N tissue were considered to be somatic (Table S1). In patient F1 (CAS), 46 filtered somatic variants were found in T but not in N cardiac tissue (Table 2) including an in-frame deletion in the KDR gene (p.Asn704del), which encodes VEGF receptor 2 (VEGFR2), which belongs to the VEGF-angiogenesis signaling pathway (Figure 3A and 3B).

Regarding the F2 individual, only 13 filtered somatic variants were found in the T tissue of the breast AS
Table 1. Total Cases and Number of Variants Found in the Whole Exome Sequencing

| Individual | Pathology     | Tissue | Variant Calling | Filtered Variants | Somatic Variants* | Constitutional Variants† |
|------------|---------------|--------|-----------------|-------------------|-------------------|-------------------------|
| *POT1* p.Arg117Cys carriers |               |        |                 |                   |                   |                         |
| F1         | CAS           | T      | 1095            | 46                | NA†               |                         |
|            |               |        |                 |                   |                   |                         |
| F2         | Papillary thyroid | T    | 100 506         | 1294              | 5                 | NA†                     |
|            |               |        |                 |                   |                   |                         |
| Breast AS | T             | 93 496 | 1276            | 13                | NA†               |                         |
| Without mutations in the *POT1* gene |               |        |                 |                   |                   |                         |
| NT1        | Sporadic CAS  | T      | 102 560         | 1266              | 62                | 1032                    |
|            |               |        |                 |                   |                   |                         |
| NT2        | Sporadic CAS  | T      | 100 120         | 1231              | 36                | 1101                    |
|            |               |        |                 |                   |                   |                         |
| NT3        | Sporadic CAS  | T      | 97 233          | 704               | 37                | 1180                    |
|            |               |        |                 |                   |                   |                         |

| Only T tissue available‡ | Individual | Pathology     | Tissue | Variant Calling | Filtered Variants | Stringent Filtering |
|-------------------------|------------|---------------|--------|-----------------|-------------------|---------------------|
| T1                      | Sporadic CAS | T             | 93 374 | 1181            | 315               |                     |
| T2                      | Sporadic CAS | T             | 95 492 | 1274            | 403               |                     |
| T3                      | Sporadic CAS | T             | 104 545| 1328            | 443               |                     |
| T4                      | Sporadic CAS | T             | 93 859 | 1233            | 361               |                     |

CAS indicates cardiac angiosarcoma; N, normal tissue; NA†, not applicable (*POT1* p.Arg117Cys carriers from Calvete et al [2015]); T, tumor tissue.

*Variants found in tumor tissue that were not found in normal tissue.
†Variants found in normal tissue that were not found in tumor tissue.
‡Individuals with only T tissue sequenced.

(Table S1). Two of them were variants annotated in the genes *PLCG1* (p.Leu752Val) and *PIK3CA* (p.Arg88Gln) belonging to the VEGF-angiogenesis pathway and previously described to be involved in different angiosarcomas and primary breast cancer.26 Another 5 somatic variants were found in the papillary thyroid T tissue from the same individual (Table S1). Three of these belonged to the VEGF-angiogenesis pathway (*PIK3R6* [p.Arg59Lys], *RASSF1* [p.Arg227His], and *BRAF* [p.Val600Glu]). Overall, 32/52 (62%) of the somatic missenses were C:G>T:A changes (Table S1). Genes with mutations are shown in the angiogenesis pathway depicted in Figure 3.

Finally, only cardiac tumor tissue from patient F3 (CAS) was sequenced (Table 2). A total of 297 filtered variants were found in the tumor tissue of this patient (Table S2), including another mutation in the *KDR* gene (pThr771Arg). Overall, 60% of the somatic variants found in patient F3 were annotated as C:G>T:A changes (Table S2).

Thus, somatic mutations in the VEGF signaling pathway were found in the tumor tissues of all affected individuals carrying the *POT1* p.Arg117Cys germ line mutation. Interestingly, both studied CAS patients had mutations in *KDR*, which activates VEGF signaling to regulate angiogenesis by the MAPK/ERK and AKT/PI3K pathways (Figure 3A and 3B). MAPK/ERK regulates proliferation activity, while AKT/PI3K promotes protein synthesis. Interestingly, both molecular activities regulate the cell cycle by inhibiting senescence promotion (Figure 3C). In order to test the putative effects of the mutations found in the VEGF angiogenesis pathway, we studied the MAPK/ERK and AKT/PI3K molecular pathways by IHC with antibodies against the activated (phosphorylated) forms of ERK and S6, respectively (Figure 3A and 3B). All angiosarcomas (CAS and the breast AS tumors) did not stain with anti–P-ERK but were positively stained (>70%) with the anti–P-S6 antibody. However, papillary thyroid tumor tissue stained with both antibodies (Table 1 and Figure 2B).

In summary, somatic activating mutations of the VEGF-angiogenesis pathway were found in all studied tumor tissues of *POT1*-mutated patients independently of the tumor type (cardiac, breast, and thyroid). Somatic mutations in the *KDR* gene were found in both CAS patients (F1 and F3). In addition, positive P-S6 staining was observed in all angiosarcomas with somatic mutations in the *KDR* (CAS) and *PI3K* (breast AS) genes (AKT/PI3K pathway). The papillary tumor (F2), which had a mutation in the MAPK/ERK signaling pathway (*BRAF* gene), was stained with both anti–P-ERK and anti-PS6 antibodies (Figures 2B and 3).

Sporadic Cardiac Angiosarcoma Patients Without Mutations in the *POT1* Gene

To assess the whole genetic landscape of CAS tumors, another 7 patients with sporadic CAS tumors who were not
Figure 2. Immunohistochemical staining. A, Tissue stress was tested in normal tissue of carriers of the POT1 p.Arg117Cys (p.R117C) mutation and carriers of mutations in the damage response-signaling pathway (sporadic CAS) in comparison with the corresponding normal tissue without mutations (wild type). Above: Wild-type cardiac and thyroid tissues from healthy controls without mutations. Below: normal tissue of individual NT2 (sporadic CAS individual with constitutional mutations in the ATR, ATM, and TP53BP genes) and normal tissue of individual F2 (papillary thyroid tumor with POT1 p.R117C mutation) as representative examples (see Table 2 for all studied individuals). Increased cell cycle arrest was observed in the normal tissue of both patients. Black arrowheads show some of the stained nuclei. Detailed fields (10×) are also shown. Scale bar (in black): 100 μm. B, IHC staining with anti–P-ERK and anti–P-S6 antibodies in tumor tissues. Tumor tissues from carriers (F1 and F2) and noncarriers (T1 and NT2) of the POT1 p.R117C mutation compared with a normal tissue section (negative staining) are shown as examples (see Table 2 for all studied individuals). Three tumors from the 2 patients (F1 and F2) carrying the POT1 p.R117C mutation are shown: both angiosarcomas (CAS tumor tissue from F1 and breast AS from patient F2) only showed immunoreactivity with anti–P-S6 antibody, while the papillary thyroid tumor (patient F2) also showed immunoreactivity with anti–P-ERK antibody. Two staining patterns were observed in sporadic CAS patients without mutations in the POT1 gene: tissue from patient T1 only showed immunoreactivity with anti–P-S6, whereas tissue from patient NT2 (sporadic CAS) was stained with both anti–P-S6 and anti–P-ERK antibodies. Scale bar (in black): 100 μm. AS indicates angiosarcoma; CAS, cardiac angiosarcoma; N, normal tissue; T, tumor tissue.
carrying mutations in the *POT1* gene were studied. Normal (N) and tumor (T) cardiac tissues were available from 3 sporadic CAS individuals (NT1, NT2, and NT3), whereas only tumor tissue was available from the other 4 CAS individuals (T1 to T4). We found 1032, 1101, and 1180 constitutional variants in cardiac tissue for the NT1, NT2, and NT3 CAS individuals, respectively; 62, 36, and 37 somatic variants were found for individuals NT1, NT2, and NT3, respectively (Table 2).

Regarding the 4 tumor samples of which only T tissue was available, no distinction could be made between constitutional and somatic variants. We found 1181, 1274, 1328, and 1233 variants in the T tissue of patients T1, T2, T3, and T4, respectively (Table 2).

To further delineate the genetic landscape of sporadic CAS tumors, the genes encompassing filtered variants were grouped into 2 different pathway enrichment analyses. The first set included genes with constitutional (found in both N and T tissues from cases NT1, NT2, and NT3) and all genes with variants from the other 4 CAS individuals with only the tumor tissue sequenced (constitutional or somatic) (2501 unique genes in total); the second set included the genes with somatic variants (only present in T tissue) from cases with N and T tissue (NT1, NT2, and NT3) and again all genes with variants from the other 4 CAS individuals with only the sequenced T tissue cases (T1 to T4) (1522 unique genes in total).

**Constitutional Events in Normal Tissue in Sporadic CAS**

This study revealed that the most represented pathway and the pathway with the major number of genes were the “Sustainability of p53 pathway” (genes *ATM, TP53, and RFWD2*) (*P* value 0.003) and the “Repair modulation pathway” (genes *ATR, ATM, TP53, RFWD2, SIRT7, BRCA2, CDK8, UBE2D1, WRN, PMS2, BRIP1, TP53BP2, and APC2*) (*P* value 0.00022), respectively (Table S3). Mutations in genes from these pathways were found in all 7 sporadic CAS individuals (Figure 4). These genes belong to the damage response signaling pathway and may deregulate the cell cycle in the same manner as previously observed for the *POT1* mutation carriers. Thus, N cardiac tissue from the sporadic CAS individuals (NT series) was also stained with anti–P-γH2AX, anti-p21, and anti-p27 antibodies. Positive staining was also observed in N tissue of sporadic CAS individuals (Table 1), which correlated with cell cycle deregulation as observed in familial angiosarcomas (*POT1* p.Arg117Cys mutation carriers). Therefore, the familial angiosarcomas (*POT1* p.Arg117Cys carriers) and sporadic CAS patients behaved in a similar way regarding cell cycle arrest regulation.

**Somatic Events in Tumor Tissue in Sporadic CAS**

A second pathway enrichment analysis was performed with the gene set including the somatic variants found in T tissues of sporadic CAS individuals (see above). This enrichment analysis revealed that the most represented pathway was the “gf-hypoxia and angiogenesis” pathway (Biocarta: 16.7%) (*P* value 0.00557). The pathway with the major number of affected genes was the “Signaling by VEGF” pathway (Reactome, 24 genes) (*P* value 0.00101) (Table S3). Both enrichment analyses corresponded with the VEGF-angiogenesis pathway. Genes with mutations are shown in the angiogenesis pathway of Figure 3. Mutations in genes from these pathways were found in all 7 sporadic CAS individuals (Figure 4). Activation of the MAPK/ERK and AKT-PI3K pathways was studied by IHC with anti–P-ERK and anti–P-S6 antibodies, respectively.

Tumors of all studied sporadic CAS individuals (NT and T series) stained positive with anti-PS6 antibodies, which demonstrates that somatic mutations were activating the VEGF-angiogenesis pathway (Table 1). Especially intense staining was also observed in the endothelial lining of blood vessels. However, not all tissues from individuals affected with sporadic CAS stained with anti-PERK antibody. Tissue of

| Mutation | Sample | Pathology | Tissue | N Tissue | γH2AX | p21 | p27 | Mutations in VEGF-Angiogenesis Pathway | T Tissue | P-ERK | P-S6* |
|----------|--------|-----------|--------|----------|-------|-----|-----|------------------------------------------|----------|-------|-------|
| *POT1*   | F1     | CAS       | Cardiac (N+T) | +       | +     | +   |                                 | KDR      | -     | +     |
| p.Arg117Cys | F2  | Breast AS | Breast T | NA   | NA   | NA   |                                 | PI3K     | -     | +     |
|          |        |           | Thyroid (N+T) | +   | +   | +   |                                 | BRAF     | +     | +     |
| DR genes | NT2    | CAS       | Cardiac (N+T) | +       | +     | +   |                                 | VEGF2/RAS-MAPK | +     | +     |
| T1       | CAS    | Cardiac (T) | NA   | NA   | NA   |                                 | Akt-PI3K | -     | +     |
| T3       | CAS    | Cardiac (T) | NA | NA   | NA   |                                 | Ras-MAPK/Akt-PI3K | +     | +     |
| T4       | CAS    | Cardiac (T) | NA | NA | NA |                                 | VEGF/Akt-PI3K | +     | +     |

*Indicates overexpression; −, no expression; CAS, cardiac angiosarcoma; DR, damage response; N, normal; NA, tissue not available; T, tumor.

*Positive staining in lining epithelium and tissue.

Table 2. Immunohistochemistry Staining Results for the Studied Cases

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individual T1, who only had mutations in the AKT-PI3K signaling pathway (Table 1), did not stain with anti-P-ERK. The tumors with mutations in the 2 molecular signaling pathways (NT1, NT2, and T4) also stained positive with the 2 antibodies (anti-P-ERK and anti-P-S6) (Table 1). Interestingly, the individual with mutations only in the MAPK/ERK signaling pathway (T3) also stained positive with both antibodies (Table 1). Stained tissues from patients T1 and NT2 are shown in Figure 2B as representative examples.

In summary, all studied individuals with CAS (either familial or sporadic) had mutations (either constitutional or somatic) in normal tissue affecting damage response signaling (Figure 4). IHC with anti-p21 and anti-p27 antibodies confirmed cell cycle arrest deregulation in N tissue that leads to constitutional cell cycle arrest and cessation of cell division (Figure 2A and Table 1). In addition, somatic activating mutations in the VEGF-angiogenesis pathway were found in tumor tissue of familial and sporadic angiosarcomas, independently of the presence of POT1 mutations (Figures 2B and 4; Table 1).

Sequencing Replication With the Truseq170 Panel and IBM Watson for Genomics Platform

A sequencing replication was performed for 2 previously sequenced CAS patients without mutations in the POT1 gene. The Truseq170 panel was run for tumor tissue of the T1 and T4 individuals and analyzed with the IBM Watson for Genomics platform (version 33.148), which is a novel sequencing platform that predicts the most probable variant causing the pathology and provides suggestions for translating the data to the clinic. The Watson for Genomics pipeline revealed 15 variants of unknown significance, 2 alterations without proposed therapies, and only 1 actionable alteration for patient T1 (Table S4). Three variants of unknown significance, 3 alterations with no proposed therapies, and another 3 actionable alterations were described for patient T4 (Table S5). Interestingly, a not previously detected copy number gain was found for the KIT gene in the tumor tissue of patient T1. The gained region is involved in tumor cell proliferation, angiogenesis, and metastatic disease. Only 1 actionable pathway was found in common for both CAS patients. The variants found in the TP53 gene were highlighted as actionable alterations, as previously found in the WES study (TP53 p.Arg175His and TP53 p.Val143Met for patients T1 and T4, respectively).

The single-strand DNA response in telomeres is inactivated by the shelterin complex (Figure 1A and 1B). We previously observed that the POT1 p.Arg117Cys mutation prevented the POT1 protein from binding to TPP1 and forming the OB-fold to bind single-strand DNA, which led to abnormally long telomeres. Here, we observed that abnormal telomere length found in our patients correlates with a genomic instability scenario that, in consequence, activates DNA damage-signaling (γH2AX-positive staining). Individuals carrying the POT1 p.Arg117Cys mutation overexpressed the p21 and p27 proteins in constitutional tissue (Table 1), which correlates with prevention of the repression of ATR signaling and leads to cell cycle arrest (Figure 1D). This increased senescence in nontumor tissue would result in reduced cell cycling and cessation of cell division. A similar mechanism was proposed to explain how short telomeres can lead to vascular senescence and diminished proliferative capacity that involved exhaustion of cell pools in mice.

In addition, tumor tissue of POT1 p.Arg117Cys mutation carriers was studied in order to evaluate the involvement of constitutional cell cycle arrest in tumor progression. On average, 61% of the somatic variants found in these patients were C>G>T:A changes (Tables S1 and S2). At this time we cannot rule out a correlation between this observed bias and a specific mutation signature in these tumors. Megquier et al have established an association with somatic deamination of cytosine to thymine in a large series of angiosarcomas (different from cardiac), which is in agreement with our observation. Somatic mutations in the VEGF-angiogenesis pathway were found in tumor tissue of all individuals (Figure 4). Interestingly, the F1 and F3 individuals (CAS) also had a somatic mutation in the KDR gene, which is involved in VEGF/PLCG1 activation and was described altered in 2 recently studied sporadic CAS cases. In the F2 patient the recurrent somatic mutations PIK3CA p.Arg88Gln and BRAF p.Val600Glu were found in the breast AS and the papillary thyroid tumor, respectively. Twenty-five percent of all breast cancers have somatic mutations in the PIK3CA gene. Specifically, the PIK3CA p.Arg88Gln mutation was described in a primary breast cancer. The somatic mutation BRAF p.Val600Glu is the most common genetic change in papillary thyroid cancers (35.8%). In addition, the
**BRAF** p.Val600Glu and **PIK3CA** p.Arg88Gln mutations have been described as activators of the angiogenic response.\textsuperscript{31,32} The somatic mutation **BRAF** p.Val600Glu has also been reported in gliomas\textsuperscript{33} and melanomas,\textsuperscript{34} where constitutional mutations in the **POT1** gene were also described.\textsuperscript{10-12} In summary, individuals with nonfunctional **POT1** did not repress damage signaling (ATR) and showed a constitutive increase of p21 and p27 expression, which is in agreement with cell cycle arrest. In addition, somatic activating mutations in the angiogenesis pathway were acquired. Interestingly, activating somatic mutations in the **KDR** gene were found in both studied patients affected with CAS and carrying a **POT1** germline mutation (F1 and F3). This was also observed in other tissues and tumors different from cardiac angiosarcomas with mutations in the **POT1** gene (thyroid and breast from patient F2), demonstrating that not only in cardiac tissue is there a strong correlation between constitutional senescence mediated by **POT1** malfunction and the acquisition of somatic mutations in the angiogenesis pathway.

**Figure 3.** VEGF (vascular endothelial growth factor)-angiogenesis signaling and cell cycle regulation pathways. VEGF signaling is a growth factor pathway to stimulate vasculogenesis and angiogenesis. A, MAPK/ERK signaling regulates cell proliferation. B, AKT/PI3K signaling is related to protein synthesis and cell cycle signaling. Locations of anti–P-ERK and anti–P-S6 used in IHC studies are also shown in the pathway (white arrows). C, Damage-signaling pathway. AKT/PI3K signaling inhibits apoptosis, senescence, and cell cycle arrest through inhibition of FOXO, which in turn positively regulates p21 and p27 activity.

**Sporadic Cardiac Angiosarcomas Without **POT1** Mutations**

Although in 4 of the 10 patients studied no discrimination between germline and somatic mutations could be made, mutations in the pathways that were affected by the mutation in the **POT1** gene at both the constitutional and the somatic level were also found in these patients.

Studyed CAS individuals without constitutional mutations in the **POT1** gene presented mutations in genes that were involved in damage response signaling (ATM-ATR-TP53) (Figure 4). IHC studies with anti-p21 and anti-p27 antibodies confirmed the damage response activation in all sporadic CAS individuals (Table 1 and Figure 2). Therefore, our results suggest a relation between telomere instability (familial CAS) and altered damage signaling (sporadic CAS) on 1 hand, and increased cell cycle arrest leading to the cessation of cell division on the other. Our observation is in agreement with previous studies in which overexpression of TP53 was detected by immunohistochemistry in 49% of angiosarcoma...
### Genetic Landscape of Cardiac Angiosarcomas

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**Sporadic CAS**

| Telomere instability & Damage response | N/T CAS | T CAS |
|----------------------------------------|---------|-------|
| POT1                                   |         |       |
| ATR                                    |         |       |
| BRCA1                                  |         |       |
| BRCA2                                  |         |       |
| ATM                                    |         |       |
| RAD51B                                 |         |       |
| RAD51C                                 |         |       |
| TP53                                   |         |       |
| TP53RK                                 |         |       |
| TB53BP1                                |         |       |
| TB53BP2                                |         |       |
| APC2                                   |         |       |
| CDK8                                   |         |       |
| BRIP1                                  |         |       |

**VEGF-angiogenesis**

| KDR                                    |         |       |
| VEGFA                                  |         |       |
| FGF22                                  |         |       |
| DOCK1                                  |         |       |
| VAV3                                   |         |       |
| SPTAN1                                 |         |       |
| SRC                                    |         |       |
| SOS1                                   |         |       |
| RASA2/4                                |         |       |
| RASGEF1A                               |         |       |
| NRAS                                   |         |       |
| RASGEF1A                               |         |       |
| ITGAV                                  |         |       |
| IQGAP1                                 |         |       |
| MAP2K1                                 |         |       |
| MAP2K3                                 |         |       |
| PPP2R5E                                |         |       |
| PTPRQ                                  |         |       |
| BRAF V600E                             |         |       |
| PDK1                                   |         |       |
| PLCG1                                  |         |       |
| PRKCC                                  |         |       |
| NRG2                                   |         |       |
| PIK3C2G                                |         |       |
| PI3K                                   |         |       |
| RICTOR                                 |         |       |
| SHOX2                                  |         |       |
| DUSP8                                  |         |       |

- **confirmed constitutional mutation**
- **confirmed somatic mutation**
- **confirmed somatic mutation (less stringent filtering)**
- **Mutation (only tumor tissue studied)**
- **Mutation (only tumor tissue studied) (less stringent filtering)**

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Constitutional Cell Cycle Arrest May Fuel the Acquisition of Somatic Mutations in the Angiogenesis Pathway in Angiosarcomas

Activation of damage signaling in both familial (POT1 mutations carriers) and sporadic angiosarcomas (ATM-ATR-TP53 mutation carriers) induced constitutional senescence and reduced cell division (Figure 2). Our results strongly suggest a correlation between constitutional cell cycle arrest and the acquired somatic mutations in the VEGF-angiogenesis pathway that drive angiosarcoma formation. Moreover, increased cell cycle arrest due to POT1 malfunction also uncovered somatic angiogenesis activation in tumors other than cardiac angiosarcomas. Damage response signaling and the VEGF-signaling pathway are mutually regulated (Figure 3). Recently, telomere biology and the PI3K pathway (angiogenesis) were also shown to be functionally connected, and phosphorylation activity of the PI3K/AKT pathway was demonstrated to affect telomere stability in vitro. In vitro studies with stem cells from Pot1a knockout mice with increased telomere dysfunction also suggested a correlation with increased proliferation. Our results indicate that the observed cell cycle deregulation may interfere with apoptosis. This bypass of apoptosis would permit the acquisition of somatic mutations. In addition, cells carrying somatic mutations in genes involved in attenuating cell cycle arrest, which depletes progenitor stem cells in nontumor tissues, may undergo positive selection. A bypass of apoptosis was also suggested in studies in which POT1 was inactivated in vitro, and induced genomic instability enabled cancer cells to acquire additional mutations and conferred aggressive behavior. Attenuation of the damage response was suggested to allow tumor cells to bypass the proliferation defect imposed by POT1 inhibition. Therefore, under senescence conditions, activating somatic mutations in the angiogenesis pathway would acquire an important role to replenish the depleted tissue. However, the mutations found in the angiogenesis pathway would contribute to angiosarcoma formation and progression.

Clinical Significance of the Identified Mechanism

Activation of the VEGF-angiogenesis pathway was found in all studied individuals with AS (familial and sporadic). However, our IHC studies revealed 2 different staining patterns that correlated with the location of the somatic mutations (Figure 2). Tumors with mutations in the AKT-PI3K signaling pathway (F1, F2, F3, and T1) were only positively stained with the anti-P-S6 antibody (Table 1 and Figure 4). The individuals with mutations only in the MAPK/ERK signaling pathway (NT1, NT2 and the papillary thyroid tumor of F2) or in both molecular signaling pathways (T3 and T4) were positively stained with both antibodies (Table 1). Therefore, different somatic alterations regarding increased angiogenesis may occur in response to senescence. These results give important clues regarding the diagnosis and classification of angiosarcomas.

Our results also have an important clinical relevance regarding treatment and translational research. Inhibition of angiogenesis may be useful to stop tumor progression. However, angiogenesis inhibition would mitigate the effect of the driving somatic mutations but would not revert cell cycle arrest or the suggested bypass of apoptosis and would therefore not curtail the acquisition of new somatic mutations. Treatment with PI3K inhibitors of patient-derived xenografts also showed increased telomeric DNA damage. Our results suggest that further studies regarding ATM/ATR, damage signaling and cell cycle inhibition activity might lead to recovering cell cycle control and preventing the acquisition of somatic mutations, including in asymptomatic patients carrying POT1 mutations. Regarding this issue, the second
sequencing experiment with the IBM Watson platform also pointed to the damage-signaling pathway as a therapeutic target. Actionable variants in the TP53 gene were highlighted in both studied CAS individuals (T1 and T4). Therefore, although somatic driver variants were found to occur in the angiogenesis pathway, only damage response signaling was found actionable for both studied angiosarcomas (Tables S4 and S5).

In summary, our current results demonstrate that inhibition of POT1 gene function and damage response malfunction both activate ATR-dependent DNA damage signaling, which increases cell cycle arrest that would diminish cell proliferation in constitutional tissue, and that triggers somatic activating mutations in the angiogenesis pathway in angiosarcomas. Interestingly, our results and the 2 previously studied CAS patients suggest a strong correlation between constitutional mutations in the POT1 gene, somatic activating mutations in the KDR gene, and CAS development. Importantly, the same mechanism was observed in tumor types different from cardiac tumors for patients carrying POT1 mutations and long telomeres (Figure 2). The significance of this mechanism needs to be further evaluated, and it is conceivable that POT1 mutations lead to the same acquired somatic alterations in other tissues and tumor types such as glioma, melanoma, or colorectal cancer. Therefore, mutations found in the POT1 gene and other genes involved in DNA damage-response signaling (ATR/ATM and TP53) in the studied cardiac angiosarcomas correlate with constitutional cell cycle arrest, which would deplete the progenitor cells and trigger tissue stress. This tissue stress would give rise to a bypass of the apoptotic regulation, which permits the acquisition of multiple somatic events. In all studied CAS cases (patients with familial CAS carrying the POT1 mutation and patients with sporadic CAS), somatic activating mutations were found in the angiogenesis pathway, which drives tumor formation. At a translational level, inhibition of angiogenesis might be useful to halt tumor progression. However, inhibition of angiogenesis would not reverse cell cycle arrest or the suggested bypass of apoptosis in constitutional asymptomatic tissue. Instead, the use of ATM/ATR activity inhibitors might restore cell cycle control and prevent the acquisition of somatic mutations.

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Disclosures

None.

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Table S1. Filtered somatic variants found in the WES of \textit{POT1} p.Arg117Cys carriers, one CAS and one breast AS with papillary thyroid cancer.

Genes previously shown to be involved in the corresponding pathology are highlighted in grey.

| Pathology | Chr | Gene   | Position   | REF     | ALT | ALT allele fraction | Amino acid change | dbSNP       | Cosmic      | GMAF | ExAC |
|-----------|-----|--------|------------|---------|-----|---------------------|------------------|-------------|-------------|------|------|
| CAS       | 1   | TMEM52 | 1850627    | CAGCGGCAGG | C   | 0.224           | L26del          | COSM1167507 | -            | -    | -    |
|           | 1   | RCC1   | 28858834   | G       | A   | 0.184           | G169Asp         |             |             | -    | -    |
|           | 1   | CELSR2 | 109792751  | C       | C   | 0.432           | L17P            | rs200277265 | COSM1200708 | -    | -    |
|           | 1   | NOTCH2 | 120612234  | G       | C   | 0.234           | -               |             |             | -    | -    |
|           | 1   | NOTCH2NL | 145248838 | A       | G   | 0.232           | -               | rs20120485  | -            | -    | -    |
|           | 1   | ARNT   | 150849103  | C       | A   | 0.165           | -               | rs10305649  | 0.0289   | 0.05 | -    |
|           | 1   | LOR    | 153233990  | T       | G   | 0.212           | Y189D           |             |             | -    | -    |
|           | 2   | NRXN1  | 50765449   | C       | T   | 0.346           | W735*           |             |             | -    | -    |
|           | 2   | ANKRD36| 97820478   | G       | A   | 0.432           | c.1260+3D       | rs79756591  | COSM1632174, | -    | -    |
|           | 2   | RABL2A | 114386168  | C       | T   | 0.218           | -               |             |             | -    | -    |
|           | 2   | HS6ST1 | 129075877  | G       | T   | 0.214           | D87E            |             |             | -    | -    |
|           | 2   | HS6ST1 | 129075939  | T       | A   | 0.168           | K67*            | rs20224738  | COSM1129578 | -    | -    |
|           | 2   | HOXD11 | 176972061  | G       | C   | 0.344           | -               |             |             | -    | -    |
|           | 2   | SPATA3 | 231861032  | TCAGCGCCCTAGCCCTGAATCCACACCA | T | 0.188           | Q30_Q38del     | rs72362780  | COSM1406147 | -    | -    |
|           | 2   | NGEF   | 233748132  | C       | T   | 0.234           | R549H           |             |             | -    | -    |
|           | 3   | FANCD2 | 10088266   | G       | T   | 0.434           | c.1137G>T+3D    | rs72492997  | -            | -    | -    |
|           | 4   | KDR    | 55968550   | CATT    | C   | 0.424           | N704del         |             |             | -    | -    |
|           | 4   | ALB    | 74280882   | G       | A   | 0.546           | V397M           |             |             | -    | -    |
|           | 4   | DSPP   | 88537069   | T       | TGATA GCAGC | 0.288 | S1085_insDSS     |             |             | -    | -    |
|   | Gene   | GenBank Accession  | Chromosome | Start | End | Mutation Description | rsN | Other Information |
|---|---------|--------------------|------------|-------|-----|----------------------|-----|------------------|
|4  | CENPE   | 104119549          | 10         | 1486  | 2139| G>C 0.212            | -   | -                |
|4  | SYNP02  | 119951749          | 4          | 2930  | 3300| T>C 0.188 R607*      | -   | -                |
|4  | CBR4    | 169931326          | 4          | 1732  | 1965| C>G 0.434            | -   | rs67305871       |
|6  | ATAT1   | 30595639           | 4          | 825   | 1005| G>C 0.465 G79A       | -   | -                |
|6  | HLA-DRB5| 32497905           | 6          | 1069  | 1131| A>G 0.365 R33*       | rs71549219 | -                |
|6  | HBS1L   | 135290447          | 6          | 753   | 845 | T>G 0.434 E609D      | -   | -                |
|7  | ZAN     | 100385561          | 5          | 1707  | 1829| A>C 0.708            | -   | COSM1329481, COSM1329482, COSM1329483 |
|7  | TRBV5-5 | 142148969          | 11         | 3327  | 3386| A>T 0.498 L101*      | -   | -                |
|10 | ARMC4   | 28250610           | 10         | 1152  | 1216| A>G 0.370 D425Y      | -   | -                |
|10 | FGFR2   | 123325217          | 10         | 936   | 1156| C>T 0.430 c.1111-1    | -   | -                |
|11 | MUC6    | 1030228            | 11         | 97    | 132 | A>C 0.708 C334G      | rs20098033 | COSM1603961, 0 COSM1603962 |
|11 | CLCF1   | 67141148           | 11         | 2044  | 2111| AT>A 0.278           | -   | -                |
|11 | PDE2A   | 72301235           | 11         | 735   | 826 | CGT>C 0.234 Y252SfsTer74 | -   | -                |
|12 | DDX11   | 31237978           | 12         | 918   | 1009| C>T 0.188 R186W      | rs74087925 | -                |
|12 | PRKAG1  | 49397570           | 12         | 868   | 982 | C>A 0.234 V234F      | -   | -                |
|12 | KMT2D   | 49434325           | 12         | 721   | 811 | G>A 0.436 R2410*      | -   | COSM144609, COSM1299436 |
|12 | KMT2D   | 49444088           | 12         | 385   | 461 | GGA>G 0.298 L1094PfsTer20 | -   | -                |
|14 | ZFP36L1 | 69256806           | 14         | 120   | 157 | T>G 0.436 K154T       | -   | -                |
|16 | TOX3    | 52484238           | 16         | 1235  | 1415| G>A 0.186 Q31L       | -   | -                |
|16 | PSMB10  | 67970188           | 16         | 186   | 202 | G>C 0.688 R58G       | COSM139019 | -                |
|17 | SHPK    | 3524660            | 17         | 2304  | 2403| CGGCAT>C 0.444 Ii30_Ai31del | -   | -                |
|19 | RHPN2   | 33490566           | 19         | 251   | 280 | G>T 0.212 Q384R      | -   | -                |
|19 | RHPN2   | 33490585           | 19         | 293   | 320 | G>A 0.388 Q378*      | rs78615454 | COSM1318333 |
|22 | POTEH   | 16287784           | 22         | 353   | 402 | C>T 0.268 W34*       | rs20037519 0 | 0.006 |
|22 | SRRD    | 26879946           | 22         | 144   | 169 | GGAGGCATC>G 0.160 A133_A39del | rs66831137 | -                |
| X | Gene  | Sample Size | Chromosome | Ref | Alt | p-value | SNP | HGNC | Symbol | BeadArray ID | p-value | Symbol |
|---|-------|-------------|-------------|-----|-----|---------|-----|-------|---------|--------------|---------|---------|
| 3 | PIK3CA | Breast AS | 178916876 | G   | A   | 0.348   | R88Q|       |         | rs12191328  |         |         |
| 4 | TRIM2  |          | 154217059 | G   | A   | 0.656   | V461M|       |         |             |         |         |
| 5 | EIF4E1B|            | 176070736 | G   | A   | 0.655   |       |       |         |             |         |         |
| 7 | BRAF  | Thyroid   | 140453136 | A   | T   | 0.324   | V600E|       |         | rs11348802  |         |         |
| 11 | CBL    |           | 119148966 | T   | G   | 0.212   | C396G|       |         | COSM34074   |         |         |
| 12 | VWF    |            | 6103147  | T   | C   | 0.214   | Y2160C|       |         |             | <0.0001 |         |
| 13 | PARP4  |            | 25021323 | A   | G   | 0.344   | I1039T|       |         | rs73172125  |         | COSM147647|
| 15 | CSPG4  |            | 75982085 | C   | T   | 0.156   | E441K|       |         | rs79463888  |         | COSM1317754|
| 20 | PLCG1  |           | 39795452 | C   | G   | 0.434   | L752V|       |         |             |         |         |
| 20 | WFDC8  |           | 44180670 | G   | A   | 0.344   | R241C|       |         | rs14756052  |         | COSM1027261|
| 3  | RASSF1 | Papillary | 50369082 | C   | T   | 0.212   | R227H|       |         |             |         |         |
| 6  | MDGA1  |           | 37606032 | C   | T   | 0.234   | V909I|       |         | COSM1600157 |         |         |
| 6  | ARID1B |           | 157528658| G   | A   | 0.168   | R2168Q|       |         |             | <0.0001 |         |
| 17 | PIK3R6 |           | 8741894 | C   | T   | 0.254   | R59K |       |         |             | <0.0001 |         |
Table S2. Filtered variants found in the WES of F3 patient affected with CAS (*POT1* p.R117C carrier).

| Chr | Gene Symbol | Genomic Position | REF | ALT | Fraction | HGVSp | Existing Variation | GMAF |
|-----|-------------|------------------|-----|-----|----------|-------|-------------------|------|
| chr1 | KYAT3       | 88949093         | A   | T   | 0.128    | ENSP00000260508.4:p.Leu380Gln | rs144984854 | 0.0006 |
| chr2 | ANKRD36     | 97211741         | G   | A   | 0.436    | ENSP00000391950.2:p.Val1157Met | rs10194525 | 0.0006 |
| chr6 | HLA-DRB1    | 32580249         | C   | G   | 0.310    | ENSP00000353099.5:p.Thr262Arg | rs199727427 | 0.0006 |
| chr10| EEF1AKM     | 124774782        | C   | T   | 0.168    | ENSP00000357829.2:p.Ala98Thr | rs199727427 | 0.0006 |
| chr13| PARP4       | 24447185         | A   | G   | 0.304    | ENSP00000371419.3:p.Ile1039Thr | rs199727427 | 0.0006 |
| chr17| MYH13       | 10306461         | G   | A   | 0.344    | ENSP00000404570.3:p.Arg1822Trp | rs116935297 | 0.007  |
| chr17| MAP2K3      | 21300875         | G   | T   | 0.456    | ENSP00000345083.4:p.Arg94Leu | rs56067280 | 0.0006 |
| chr18| ANKRD30B    | 14843024         | C   | G   | 0.478    | ENSP00000351875.4:p.Pro918Ala | rs180690700 | 0.0006 |
| chr19| COLGAL1T1   | 17560463         | G   | C   | 0.268    | ENSP00000252599.3:p.Leu163Val | rs76429704 | 0.0006 |
| chr17| VPS13D      | 12249234         | A   | C   | 0.677    | ENSP00000478104.1:p.Gln153His | rs11641583 | 0.0018 |
| chr17| FAM131C     | 16058547         | G   | A   | 0.104    | ENSP0000034814.4:p.Arg245Trp | rs77667563 | 0.0034 |
| chr17| CROCC       | 16940041         | C   | G   | 0.126    | ENSP00000364691.4:p.Asp586His | rs9435714 | 0.0034 |
| chr17| KDF1        | 26952098         | A   | G   | 0.456    | ENSP00000319179.5:p.Cys95Arg | rs148853297 | 0.0034 |
| chr17| PPCS        | 42456962         | A   | T   | 0.546    | ENSP00000361642.3:p.Arg133Trp | rs199807362 | 0.0034 |
| chr17| IPP         | 45716925         | G   | A   | 0.344    | ENSP00000379739.3:p.Arg427Cys | rs142095376 | 0.0002 |
| chr17| PODN        | 53078994         | G   | A   | 0.778    | ENSP00000308315.5:p.Arg543His | rs6199355 | 0.0034 |
| chr17| FAM151A     | 54619889         | C   | G   | 0.128    | ENSP00000306888.2:p.Lys79Asn | rs114883650 | 0.0144 |
| chr17| RBMXL1      | 88983615         | C   | G   | 0.376    | ENSP0000046099.1:p.Gly71Ala | rs111779380 | 0.0004 |
| chr17| TSPAN2      | 115072962        | G   | C   | 0.201    | ENSP00000358529.2:p.Gly39Arg | rs147800870 | 0.0004 |
| chr17| PDE4DIP     | 149005208        | C   | T   | 0.567    | ENSP00000358363.4:p.Arg1396Trp | rs2798901 | 0.0004 |
| chr17| FLG2        | 152351277        | G   | A   | 0.234    | ENSP00000373370.4:p.Ser217Phe | rs201200591,COSM896166 | 0.0004 |
| chr17| CRNN        | 152409620        | A   | G   | 0.198    | ENSP00000271835.3:p.Ser488Pro | rs72689400 | 0.0058 |
| chr17| GPA33       | 167068982        | C   | A   | 0.304    | ENSP00000356842.3:p.Val119Phe | rs72689400 | 0.0058 |
| chr | Gene   | Genomic Position | Allele | Minor Allele | Minor Allele Frequency | Protein Change | Reference SNP IDs | p-value |
|-----|--------|------------------|--------|--------------|------------------------|----------------|-------------------|---------|
| chr1 | KIF14  | 200592110        | T      | G            | 0.567                  | ENSP00000356319.4:p.Lys928Thr | rs150766596 |
| chr1 | PKP1   | 201322032        | G      | A            | 0.436                  | ENSP00000263946.3:p.Glu489Lys  | rs748085816 |
| chr1 | OBSCN  | 228350867        | C      | G            | 0.128                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706, COSM5042798, COSM5042799, COSM3360727, COSM3360726, COSM3360725 | 0.0012 |
| chr1 | RHOU   | 228743354        | G      | A            | 0.436                  | ENSP00000355652.3:p.Val131Met  | rs142615706 |
| chr1 | EDARADD| 236482309        | C      | T            | 0.398                  | ENSP00000352604.2:p.Cys132Tyr  | rs142615706 |
| chr1 | OR2T3  | 248473745        | G      | A            | 0.300                  | ENSP00000356319.4:p.Lys928Thr  | rs142615706 |
| chr1 | OR2T34 | 248573866        | G      | A            | 0.126                  | ENSP00000356319.4:p.Lys928Thr  | rs142615706 |
| chr1 | OR2T27 | 248650526        | T      | C            | 1.000                  | ENSP00000356319.4:p.Lys928Thr  | rs142615706 |
| chr2 | MBOAT2 | 88732994         | C      | T            | 0.107                  | ENSP00000356319.4:p.Lys928Thr  | rs34573615 |
| chr2 | ASB3   | 53765485         | G      | C            | 0.243                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr2 | TSPYL6 | 54255416         | G      | A            | 0.778                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr2 | USP34  | 61278194         | G      | A            | 0.166                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr2 | ANKRD36C| 95944662        | T      | A            | 0.214                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr2 | ASTL   | 96132701         | C      | T            | 0.463                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr2 | NIFK   | 121732168        | C      | T            | 0.184                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr2 | LCT    | 135817693        | T      | A            | 0.564                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr2 | LRP1B  | 140868259        | G      | A            | 0.804                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr2 | NMI    | 151275501        | G      | A            | 0.756                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr2 | SLC39A10| 195716880       | A      | G            | 0.278                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr2 | TNS1   | 217882350        | A      | C            | 0.674                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr2 | ESPNL  | 238131340        | A      | G            | 0.201                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr3 | FANCD2 | 10065867         | G      | C            | 0.436                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr3 | TTC21A | 39138609         | G      | A            | 0.678                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr3 | DNAH1  | 52360039         | G      | A            | 0.322                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
| chr3 | ZNF717 | 75737092         | C      | T            | 0.128                  | ENSP00000455507.2:p.Arg702Gly   | rs142615706 |
chr3  ZNF717  75737101 G  T  0.201  ENSP00000419377.1:p.Pro791His  rs79138891
chr3  ZNF717  75737127 A  C  0.128  ENSP00000419377.1:p.His782Gln  rs79811623
chr3  ZNF717  75737622 T  A  0.344  ENSP00000419377.1:p.Arg617Ser  rs76175438
chr3  ZNF717  75737840 C  T  0.166  ENSP00000419377.1:p.Arg300Cys  rs1962893
chr3  OR5K1  98469710 T  C  0.768  ENSP00000373193.2:p.Leu45Ser  rs200654905
chr3  BBX  107716685 C  T  0.924  ENSP00000319974.8:p.Arg81Trp  rs142400819
chr3  ARHGEF26  154225981 C  G  0.440  ENSP00000348828.4:p.Asn687Lys  0.003
chr3  GPR149  154428985 G  C  0.778  ENSP00000374390.2:p.Pro211Ala  rs77408990
chr3  MUC20  195726080 C  G  0.128  ENSP00000417498.3:p.Asp493His  rs77408990
chr3  MUC4  195762138 C  A  1.000  ENSP00000417498.3:p.Ala4821Ser  CM066583
chr4  IDUA  987896 C  G  0.134  ENSP000004217498.3:p.Thr574Ile  0.0012
chr4  HTT  3207344 G  A  0.310  ENSP00000417498.3:p.Ala4821Ser  rs77408990
chr4  TLR1  38796634 G  A  0.780  ENSP00000417498.3:p.Thr574Ile  rs5743621
chr4  KDR  55098758 C  G  0.654  ENSP00000417498.3:p.Thr574Ile  rs5743621
chr4  UGT2B15  68670105 T  G  0.388  ENSP00000417498.3:p.Thr574Ile  rs200638397
chr4  RUFY3  70806627 C  G  0.124  ENSP00000417498.3:p.Thr574Ile  rs754675264
chr4  CCSE1  90308466 G  C  0.432  ENSP00000425040.1:p.Ser671Cys  rs148775298
chr4  PDLIM5  94455353 A  G  0.567  ENSP00000417498.3:p.Thr574Ile  rs754675264
chr4  UNC5C  95170263 C  T  0.128  ENSP00000417498.3:p.Thr574Ile  rs34585936
chr4  QRFPR  121380442 A  C  0.214  ENSP00000417498.3:p.Thr574Ile  rs34270076
chr4  CBR4  169002197 C  A  0.678  ENSP00000303525.3:p.Val137Phe  rs34270076
chr4  CBR4  169002200 T  A  0.455  ENSP00000303525.3:p.Val137Phe  rs34270076
chr5  TLR3  186084674 C  A  0.134  ENSP00000419377.1:p.Pro791His  rs201540925
chr5  SLC9A3  488354 C  T  0.436  ENSP00000352207.8:p.Asn131Thr  rs143027124
chr5  C5orf49  7831902 T  G  0.166  ENSP00000352207.8:p.Asn131Thr  rs143027124
chr5  ADGRV1  90705420  G  A  0.124  ENSP00000384582.2:p.Ala2803Thr  rs111033530  0.0048
chr5  MARCH3  126878360  C  T  0.356  ENSP00000309141.5:p.Arg143Gln  rs138413676
chr5  LECT2  135951324  C  T  0.304  ENSP00000274507.1:p.Gly63Glu  rs149560293  0.0068
chr5  PCDHGA6  141374360  G  A  0.256  ENSP00000231501.3:p.Glu93Lys  rs200765071
chr5  SCGB3A2  147881560  G  A  0.652  ENSP00000395538.1:p.Gly2822Arg  COSM3881739,COSM3881740
chr5  PCDHGA6  141374360  G  A  0.256  ENSP00000231501.3:p.Glu93Lys  rs200765071
chr5  PCDH12  141945665  C  T  0.732  ENSP00000274507.1:p.Gly57Asp  rs140599638
chr5  SCGB3A2  147881560  G  A  0.652  ENSP00000274507.1:p.Gly57Asp  rs140599638  0.0004
chr5  SLC36A2  151343594  C  A  0.224  ENSP00000309141.5:p.Arg143Gln  rs77010315,CM086960  0.005
chr6  GFPT2  180301596  T  C  0.364  ENSP00000274507.1:p.Gly63Glu  rs149560293
chr6  HLA  21594616  C  G  0.578  ENSP00000231501.3:p.Glu93Lys  rs140231408,COSM3697699  0.0066
chr6  BTN2A2  26392401  G  A  0.576  ENSP00000274507.1:p.Gly57Asp  rs142803339,COSM3745097  0.0092
chr6  HLA  32580779  C  T  0.128  ENSP00000231501.3:p.Glu93Lys  rs3830125
chr6  HLA  32589669  G  C  0.201  ENSP00000231501.3:p.Glu93Lys  rs148093782
chr6  HLA-DQB2  32757848  T  C  0.344  ENSP00000231501.3:p.Glu93Lys  rs9276572
chr6  PPIL1  36871822  C  G  0.134  ENSP00000231501.3:p.Glu93Lys  rs12194408  0.0098
chr6  PTK7  43130655  C  A  0.276  ENSP00000231501.3:p.Glu93Lys  rs78949718  0.0028
chr6  POLR1C  43520104  C  T  0.128  ENSP00000231501.3:p.Glu93Lys  rs148385032  0.0014
chr6  DST  56552557  C  T  0.128  ENSP00000231501.3:p.Glu93Lys  rs775546048
chr6  TTK  80035318  C  T  0.166  ENSP00000231501.3:p.Glu93Lys  rs540538876  0.0002
chr6  PNRC1  89081009  C  T  0.124  ENSP00000231501.3:p.Glu93Lys  rs2231267  0.0056
chr6  AMZ1  108953073  C  T  0.278  ENSP00000231501.3:p.Glu93Lys  rs61741320  0.0132
chr6  VNN1  132683243  T  C  0.128  ENSP00000231501.3:p.Glu93Lys  rs567530094  0.0002
chr6  SHPRH  145922832  G  A  0.243  ENSP00000231501.3:p.Glu93Lys  rs148401398  0.0014
chr7  AMZ1  2709683  G  T  0.376  ENSP00000385899.2:p.Gly441Arg  rs139796765  0.0058
chr7  SHPRH  3962743  G  A  0.455  ENSP00000385899.2:p.Gly441Arg  rs139796765  0.0058
chr7  TNRC18  5309293  C  T  0.677  ENSP00000385899.2:p.Gly441Arg  COSM3881739,COSM3881740
| chr | Gene   | Position | Ref. | Alt. | p-value | Variant ID   |
|-----|--------|----------|------|------|---------|--------------|
| chr7 | PSPH   | 56021118 | T    | C    | 0.295   | ENSP00000378854.3:p.Asp32Gly |
| chr7 | GATA1  | 92456449 | C    | T    | 0.184   | ENSP00000287957.3:p.Arg233Trp rs34768413 0.007 |
| chr7 | FBXO24 | 100590263| A    | C    | 0.128   | ENSP00000416558.2:p.Glu114Asp rs545357401 0.0002 |
| chr7 | POT1   | 124863547| G    | A    | 0.457   | ENSP00000350249.3:p.Arg117Cys rs780936436 0.0012 |
| chr7 | CPA2   | 130289628| C    | A    | 0.335   | ENSP00000322481.4:p.Leu381Met rs146796996 0.0002 |
| chr7 | TRBV20-1| 142627134| A    | T    | 0.310   | ENSP00000374917.3:p.Arg24Trp rs34768413 |
| chr7 | GATAD1 | 92456449 | C    | T    | 0.184   | ENSP00000287957.3:p.Arg233Trp rs34768413 0.007 |
| chr7 | FBXO24 | 100590263| A    | C    | 0.128   | ENSP00000416558.2:p.Glu114Asp rs545357401 0.0002 |
| chr7 | POT1   | 124863547| G    | A    | 0.457   | ENSP00000350249.3:p.Arg117Cys rs780936436 0.0012 |
| chr7 | CPA2   | 130289628| C    | A    | 0.335   | ENSP00000322481.4:p.Leu381Met rs146796996 0.0002 |
| chr7 | TRBV20-1| 142627134| A    | T    | 0.310   | ENSP00000374917.3:p.Arg24Trp rs34768413 |
| chr7 | SSPO   | 149802064| C    | T    | 0.256   | ENSP00000485256.1:p.Ser2508Phe rs140977594 0.0056 |
| chr7 | KMT2C  | 152247922| C    | T    | 0.446   | ENSP00000262189.6:p.Gly838Ser COSM4591270 |
| chr7 | MTUS1  | 17653257 | C    | G    | 0.122   | ENSP00000308720.7:p.Ile53Asn rs149246646 |
| chr7 | VIPR2  | 159031954| C    | T    | 0.788   | ENSP00000287957.3:p.Arg233Trp rs34768413 0.007 |
| chr8 | SGCZ   | 14108196 | G    | A    | 0.476   | ENSP00000371512.1:p.Pro196Leu rs145213189 0.0004 |
| chr8 | MTUS1  | 17684434 | T    | G    | 0.166   | ENSP00000262102.6:p.Lys911Thr rs147214125 0.0004 |
| chr8 | CYP7A1 | 58497114 | A    | G    | 0.436   | ENSP00000301645.3:p.Leu133Pro rs149246646 0.0004 |
| chr8 | CSPP1  | 67116017 | C    | G    | 0.657   | ENSP00000287957.3:p.Arg233Trp rs34768413 0.007 |
| chr8 | NBN    | 89971232 | G    | A    | 0.436   | ENSP0000026433.3:p.Arg215Trp rs34768413 0.007 |
| chr8 | PABPC1 | 100709584| G    | A    | 0.566   | ENSP00000313007.5:p.Arg374Cys rs200409148 0.0002 |
| chr8 | PABPC1 | 100709584| T    | C    | 0.534   | ENSP00000313007.5:p.Glu372Gly rs201076736 0.0002 |
| chr8 | PABPC1 | 100709611| C    | A    | 0.128   | ENSP00000313007.5:p.Val365Leu rs202074479 0.0002 |
| chr8 | PABPC1 | 100709584| T    | C    | 0.534   | ENSP00000313007.5:p.Arg215Trp rs34768413 0.007 |
| chr8 | DEPTOR | 119965343| G    | C    | 0.122   | ENSP00000378854.3:p.Asp32Gly 96724 0.0003 |
| Chromosome | Gene       | Position   | Allele | Minor Allele | Risk Allele | Risk Allele Effect | Minor Allele Effect | rsID            | Minor Allele Effect |
|------------|------------|------------|--------|--------------|-------------|---------------------|---------------------|------------------|---------------------|
| chr9       | ADAMTSL    | 18684733   | G      | T            | 0.184       | ENSP00000369921.4:p.Ala503Ser | 06437               | rs149221350       | COSM3906436,COSM39 |
| chr9       | LINGO2     | 27949346   | A      | C            | 0.345       | ENSP00000369328.1:p.Ile442Met |                      |                  |                     |
| chr9       | SHC3       | 89042122   | G      | A            | 0.304       | ENSP00000364995.4:p.Pro422Ser | 0771704230,COSM24411 |                  |                     |
| chr9       | OR13C5     | 104599179  | G      | A            | 0.431       | ENSP00000363911.2:p.Pro79His | 07052570,COSM4163183 |                  |                     |
| chr9       | TRIM32     | 116698344  | G      | A            | 0.128       | ENSP00000408292.1:p.Arg201His | 113259170            | 0.0012           |                     |
| chr9       | LAMC3      | 131072789  | C      | T            | 0.655       | ENSP00000354360.4:p.Ser1124Phe | 143563851,COSM4163479 | 0.001            |                     |
| chr9       | GBGT1      | 133153893  | C      | T            | 0.756       | ENSP00000367064.4:p.Ala2914Val | 45551835,COSM32707   | 0.0064           |                     |
| chr10      | CUBN       | 16890385   | G      | A            | 0.678       | ENSP00000363911.2:p.Pro79His | 143563851,COSM4163479 | 0.001            |                     |
| chr10      | GPRIN2     | 46462341   | C      | A            | 0.234       | ENSP00000363431.1:p.Val348Leu | 9426046             | 0.0012           |                     |
| chr10      | GPRIN2     | 46550598   | C      | T            | 0.436       | ENSP00000363431.1:p.Val47Met | 11204658            |                  |                     |
| chr10      | GPRIN2     | 46556022   | G      | C            | 0.234       | ENSP00000363431.1:p.Leu39Val | 140823928            | 0.0038           |                     |
| chr10      | LHPP       | 124517198  | G      | A            | 0.536       | ENSP00000357835.5:p.Asp215Asn | 374871777,COSM2021369,COSM2021368 | 0.0038          |                     |
| chr10      | CTBP2      | 124949430  | G      | A            | 0.598       | ENSP00000311825.6:p.Gly321Trp | 78155918             | 0.001            |                     |
| chr10      | CTBP2      | 124949542  | C      | T            | 0.504       | ENSP00000311825.6:p.Leu652Ile | 150320719            |                  |                     |
| chr10      | CTBP2      | 1249494563 | A      | T            | 0.544       | ENSP00000311825.6:p.Leu39Phe | 80025996,COSM5764564,COSM5764563 | 0.0038          |                     |
| chr10      | CTBP2      | 125002983  | T      | G            | 0.346       | ENSP00000311825.6:p.Asp652Ala | 796433756            |                  |                     |
| chr10      | CTBP2      | 125003006  | T      | C            | 0.310       | ENSP00000311825.6:p.Thr605Met | 112239066            |                  |                     |
| chr10      | CTBP2      | 125003010  | C      | T            | 0.215       | ENSP00000311825.6:p.Arg643Gln | 760489730,COSM5620576,COSM5620575 | 0.0038          |                     |
| chr10      | CTBP2      | 125003036  | C      | A            | 0.166       | ENSP00000311825.6:p.Lys634Asn | 201760950,COSM4441587,COSM4441586 | 0.0038          |                     |
| chr10      | CTBP2      | 125003065  | T      | A            | 0.184       | ENSP00000311825.6:p.Ile625Phe | 75794788,COSM4144456,COSM4144455 | 0.0038          |                     |
| chr10      | CTBP2      | 125003091  | G      | A            | 0.345       | ENSP00000311825.6:p.Ala616Val | 3198935              |                  |                     |
| chr10      | CTBP2      | 125003357  | G      | A            | 0.128       | ENSP00000311825.6:p.Thr605Met | 768573864,COSM2021395,COSM2021394 | 0.0038          |                     |
| chr10      | CTBP2      | 125003368  | G      | C            | 0.267       | ENSP00000311825.6:p.Asp601Glu | 1058301,COSM5763526,COSM5763525,COSM4675 | 296,COSM4675295 | 0.0038          |                     |
| Chromosome | Gene | Position (bp) | Variant | Minor Allele | Minor Allele Description | Reference SNPs |
|------------|------|---------------|---------|--------------|--------------------------|----------------|
| chr10      | CTBP2| 125003410     | C       | G            | 0.215                    | ENSP0000311825.6:p.Glu587Asp | rs74705267,COSM4144460,COSM4144459 |
| chr10      | CTBP2| 125003448     | G       | C            | 0.166                    | ENSP0000311825.6:p.Leu575Val | rs3198920       |
| chr10      | CTBP2| 125003450     | G       | A            | 0.376                    | ENSP0000311825.6:p.Pro574Leu | rs796256730,COSM5620578,COSM5620577 |
| chr10      | CTBP2| 125003460     | G       | T            | 0.435                    | ENSP0000311825.6:p.His571Asn | rs796388243       |
| chr10      | CTBP2| 125003466     | G       | C            | 0.213                    | ENSP0000311825.6:p.Pro569Thr | rs368195398       |
| chr10      | CTBP2| 125003468     | G       | T            | 0.243                    | ENSP0000311825.6:p.Gly568Val | rs368195398       |
| chr10      | CTBP2| 125003470     | G       | C            | 0.178                    | ENSP0000311825.6:p.Asn571Met | rs797010536       |
| chr11      | MUC6 | 1016961       | G       | A            | 0.166                    | ENSP0000406861.2:p.Thr1947Ile | rs773824783       |
| chr11      | MUC6 | 1017069       | G       | A            | 0.801                    | ENSP0000406861.2:p.Thr1911Met | rs80333708       |
| chr11      | MUC6 | 1017183       | A       | G            | 0.235                    | ENSP0000406861.2:p.Pro1873Gln | rs200364398       |
| chr11      | MUC6 | 1018473       | A       | T            | 0.387                    | ENSP0000406861.2:p.Pro1869Ile | rs747429892       |
| chr11      | OR4C46| 54603280 | G       | A            | 0.233                    | ENSP0000332473.1:p.Leu217Pro | rs186749084       |
| chr11      | OR4C46| 54603280 | G       | A            | 0.233                    | ENSP0000332473.1:p.Glu217Val | rs747429892       |
| chr11      | OR4C46| 54603280 | G       | A            | 0.879                    | ENSP0000329056.1:p.Ser240Phe | rs11246607       |
| chr11      | OR4C46| 54603280 | G       | A            | 0.787                    | ENSP0000329056.1:p.Ser240Phe | rs11246607       |
| chr11      | OR9G1| 56700722      | A       | G            | 0.342                    | ENSP0000309012.1:p.Tyr112Cys | rs4990194         |
| chr11      | OR9G1| 56700892      | C       | T            | 0.210                    | ENSP0000309012.1:p.Arg169Cys | rs11228733       |
| chr11      | OR9G1| 56701223      | T       | A            | 0.128                    | ENSP0000309012.1:p.Val279Glu | rs79251113       |
| chr11      | ESSENTIAL| 64315848 | T       | C            | 0.184                    | ENSP0000384851.1:p.Leu385Pro | rs201072913       |
| chr11      | ESSENTIAL| 64315856 | C       | T            | 0.376                    | ENSP0000384851.1:p.Leu388Phe | rs79204587       |
| chr11      | ESSENTIAL| 64315859 | C       | T            | 0.435                    | ENSP0000384851.1:p.Arg389Cys | rs80310817       |
| chr11      | ACTN3| 66554587      | C       | T            | 0.231                    | ENSP0000422007.1:p.Pro217Leu | rs370740496       |
| chr11      | NUMA1| 72014043      | G       | A            | 0.435                    | ENSP0000377298.3:p.Arg1154Trp | rs61740456       |
| Chr  | Gene   | Position  | Type | A | G | T | C | Frequency | Effect | rsID                |
|------|--------|-----------|------|---|---|---|---|-----------|--------|---------------------|
| chr11 | USP35  | 78210302  | G   | A | 0.768 | ENSP00000431876.1:p.Arg816His | rs75370284 | 0.0128 |
| chr11 | TRIM49C| 90041372  | C   | A | 0.465 | ENSP00000388299.1:p.Thr394Asn | rs75119043, COSM3998623, COSM3998622 | 0.0042 |
| chr11 | MAML2  | 96093510  | T   | C | 0.376 | ENSP00000434552.1:p.Gly174Asp | rs61749254 | 0.0078 |
| chr11 | SORL1  | 121496918 | G   | A | 0.346 | ENSP00000361452.2:p.Leu164Phe | rs886202 | 0.0035 |
| chr11 | OR8B2  | 124382854 | G   | A | 0.567 | ENSP00000341292.5:p.Leu313Gln | rs28919870 | 0.0004 |
| chr12 | WNK1   | 827047    | T   | A | 0.234 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0018 |
| chr12 | FOXM1  | 2858912   | G   | A | 0.128 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | CRACR2A| 3696811   | A   | C | 0.345 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | KLRC2  | 10435931  | C   | G | 0.376 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | TAS2R19| 11021703  | A   | G | 0.205 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | TAS2R19| 11031947  | G   | A | 0.345 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | TAS2R31| 11030522  | G   | A | 0.145 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | TAS2R31| 11031043  | G   | A | 0.761 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | TAS2R31| 11031194  | G   | C | 0.304 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | TAS2R31| 11134103  | G   | C | 0.166 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | PTPRO  | 15503912  | C   | G | 0.456 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | CCDC65 | 48918345  | A   | G | 0.345 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | TROAP  | 49331358  | G   | A | 0.545 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | RDH5   | 55724028  | G   | T | 0.787 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | MDM1   | 68332236  | G   | C | 0.456 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | LRRIQ1 | 85153046  | A   | C | 0.476 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr12 | DNAH10 | 123916480 | C   | A | 0.675 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr13 | MPHOSPH| 19642121  | G   | T | 0.778 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr13 | PARP4  | 24447125  | T   | C | 0.368 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr13 | PABPC3 | 25096638  | C   | T | 0.215 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| chr13 | PABPC3 | 25096659  | C   | G | 0.567 | ENSP00000341292.5:p.Leu313Gln | rs75545535 | 0.0004 |
| Chr | Gene     | Position | Ref | Alt | p-Value | Ensembl Accession | rs-ID | Custom Notes |
|-----|----------|----------|-----|-----|---------|------------------|-------|--------------|
| chr13 | PABPC3  | 25096889 | A  | G  | 0.786   | ENSP00000281589.3:p.Lys231Glu | rs78826513 |
| chr13 | PABPC3  | 25096951 | G  | T  | 0.745   | ENSP00000281589.3:p.Met251Ile | rs75281454 |
| chr13 | PABPC3  | 25097154 | C  | T  | 0.564   | ENSP00000281589.3:p.Thr319Ile | rs80261016 |
| chr13 | NEK5    | 52086336 | G  | T  | 0.786   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr13 | SLC10A2 | 103049340| C  | T  | 0.564   | ENSP00000347767.4:p.Thr319Ile | rs80261016 |
| chr14 | WIK5    | 63599636 | G  | A  | 0.124   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr14 | WDR89   | 63599677 | C  | T  | 0.266   | ENSP00000347767.4:p.Thr319Ile | rs80261016 |
| chr14 | SYNE2   | 64130105 | C  | A  | 0.340   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr14 | VIPAS39 | 77453359 | C  | T  | 0.675   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr15 | TYRO3   | 41573327 | G  | T  | 0.254   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr15 | SPATA5L1| 45403247 | C  | G  | 1.000   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr15 | SPATA5L1| 45403421 | G  | T  | 0.654   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr15 | SNX1    | 41436360 | G  | A  | 0.435   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr15 | MYO9A   | 71968075 | T  | C  | 0.675   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr16 | RPL3L   | 1947003 | C  | T  | 0.532   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr16 | ACSM2A  | 20465673 | G  | A  | 0.345   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr16 | ACSM3   | 20775918 | T  | C  | 0.546   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr16 | OR16-13 | 33827483 | G  | T  | 0.180   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr16 | CES1    | 55828971 | C  | A  | 0.184   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr16 | ZNF319  | 57996752 | C  | A  | 0.184   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr16 | AARS    | 70252728 | T  | A  | 0.215   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr16 | CLEC18B | 74409592 | C  | T  | 0.304   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr16 | ZNF469  | 88437795 | G  | C  | 0.128   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| chr17 | ITGAE   | 3757758 | G  | A  | 0.166   | ENSP00000347767.4:p.Met251Ile | rs77466429 |
| Chr | Genes | Position | Ref | Alt | SNP ID | Effect | p-value |
|-----|-------|----------|-----|-----|--------|--------|---------|
| chr17 | CTC1 | 8232058 | G | A | 0.678 | ENSP00000313759.8:p.Arg744Cys | rs35069886 |
| chr17 | MYH13 | 10309647 | C | G | 0.456 | ENSP00000404570.3:p.Asp1614His | rs35069886 |
| chr17 | MAP2K3 | 21300880 | C | T | 0.765 | ENSP00000345083.4:p.Arg96Trp | rs35069886 |
| chr17 | MAP2K3 | 21304522 | C | T | 0.678 | ENSP00000345083.4:p.Gly145Ser | rs35069886 |
| chr17 | KCNJ12 | 21415757 | G | A | 0.234 | ENSP000003463778.1:p.Glu139Lys | rs35069886 |
| chr17 | KCNJ12 | 21417755 | G | A | 0.345 | ENSP000003463778.1:p.Glu139Lys | rs35069886 |
| chr17 | KCNJ12 | 21416124 | G | A | 0.212 | ENSP000003463778.1:p.Arg261His | rs35069886 |
| chr17 | KCNJ12 | 21416127 | T | G | 0.256 | ENSP000003463778.1:p.Ile262Ser | rs35069886 |
| chr17 | KCNJ12 | 21416474 | G | A | 0.345 | ENSP000003463778.1:p.Glu378Lys | rs35069886 |
| chr17 | SPAG5 | 28578086 | T | G | 0.348 | ENSP00000323300.5:p.Asn1145Thr | rs35069886 |
| chr17 | KRTAP29-1 | 41302224 | A | T | 0.678 | ENSP00000375148.1:p.Cys210Ser | rs35069886 |
| chr17 | KRT33B | 41364989 | C | T | 0.486 | ENSP00000251646.3:p.Arg329His | rs35069886 |
| chr17 | KRT33B | 41369498 | C | T | 0.567 | ENSP00000251646.3:p.Glu85Lys | rs35069886 |
| chr17 | ETV4 | 43528697 | G | A | 0.278 | ENSP00000321835.4:p.Ala426Val | rs35069886 |
| chr17 | KANSL1 | 46171833 | T | G | 0.215 | ENSP00000387393.3:p.Lys104Thr | rs35069886 |
| chr17 | EPN3 | 50536971 | C | T | 0.676 | ENSP00000268933.3:p.Arg139Trp | rs35069886 |
| chr17 | ACE | 63491216 | C | T | 0.234 | ENSP00000290866.4:p.Thr916Met | rs35069886 |
| chr17 | CSH2 | 63872181 | T | C | 0.178 | ENSP00000376623.2:p.Glu200Gly | rs35069886 |
| chr18 | PIEZO2 | 10671669 | C | T | 0.134 | ENSP00000421377.3:p.Arg2706Gln | rs189453524 |
| chr18 | OSBPL1A | 24167436 | A | G | 0.265 | ENSP00000320291.3:p.Ser810Pro | rs35069886 |
| chr19 | PODNL1 | 13934406 | C | T | 0.234 | ENSP00000345175.4:p.Val174Met | rs781238470 |
| chr19 | PALM3 | 14053817 | C | T | 0.376 | ENSP00000344996.3:p.Asp604Asn | rs78541596,COSM1390897 |
| chr19 | WTIP | 34482629 | C | T | 0.184 | ENSP00000466953.2:p.Arg219Trp | rs78541596,COSM1390897 |
| chr19 | ZNF599 | 34769537 | C | T | 0.180 | ENSP00000338202.6:p.Asp13Asn | rs117610843 |
| chr19 | CYP2A6 | 40848628 | A | T | 0.256 | ENSP00000301141.4:p.Leu160His | rs1801272,CM980517 |
| chr19 | PPP1R37 | 45144962 | C | T | 0.324 | ENSP00000246802.4:p.Asp31His | rs78530808 |
| chr19 | NTN5 | 48664274 | G | A | 0.102 | ENSP00000270235.3:p.Ile280Thr | rs142533877 |
| chr19 | NTN5 | 48664274 | G | A | 0.102 | ENSP00000270235.3:p.Ile280Thr | rs142533877 |
chr19 LILRB2 54279040 G A 0.304 ENSP00000375629.4:p.Leu243Phe COSM1326184
chr19 LILRA2 54574799 C A 0.356 ENSP00000251377.3:p.Leu141Ile
chr19 LILRA2 54575019 A G 0.405 ENSP00000251377.3:p.Glu214Gly
chr19 KIR2DL1 54773491 C A 0.546 ENSP00000336769.5:p.Thr91Lys COSM5712917,COSM5545196,COSM3404605
chr19 KIR2DL2 54804874 A G 0.645 ENSP00000336769.5:p.Tyr53Cys
chr19 KIR3DL1 54773534 C A 0.625 ENSP00000336769.5:p.Leu243Phe
chr19 KIR3DL1 54773491 C A 0.625 ENSP00000336769.5:p.Leu243Phe
chr19 KIR3DL1 54575019 A G 0.405 ENSP00000251377.3:p.Thr91Lys
chr19 KIR3DL2 54574799 C A 0.356 ENSP00000251377.3:p.Leu141Ile
chr19 KIR3DL2 54575019 A G 0.405 ENSP00000251377.3:p.Glu214Gly
chr20 ANKRD60 58218668 G A 0.564 ENSP00000369747.1:p.Pro289Ser rs41275658
chr20 LAMA5 62317394 T C 0.678 ENSP00000216962.3:p.Tyr234His rs41275658
chr20 LAMA5 62317394 T C 0.678 ENSP00000216962.3:p.Tyr234His rs41275658
chr20 KIAA1671 25029315 A G 0.376 ENSP00000351207.3:p.Lys439Arg
chr20 CYP2D6 42127526 C T 0.128 ENSP00000353820.5:p.Arg365His rs1058172
chr20 CYP2D6 42130692 G A 0.166 ENSP00000353820.5:p.Pro34Ser CM90081
chr20 TUBGCP6 50221169 C T 0.924 ENSP00000248846.5:p.Gly1064Arg rs149231425
chr20 PLXNB2 50280899 A G 0.184 ENSP00000492273.1:p.Arg241Met
chrX PAGE1 49689423 T C 1.000 ENSP00000365320.3:p.Gly1064Arg
chrX PAGE1 49689423 T C 1.000 ENSP00000365320.3:p.Gly1064Arg
chrX FAM104B 55146247 G C 0.567 ENSP00000360671.4:p.Gly73Ser rs111638770,COSM5461083,COSM5461082,COSM388435
chrX MED12 71119383 C G 0.215 ENSP00000361933.3:p.Thr37Arg
chrX RTL9 110450737 G C 1.000 ENSP00000360671.4:p.Gly73Ser rs150383653,COSM4721270
chrX SLC25A5 119469766 G A 0.345 ENSP00000360671.4:p.Gly73Ser rs134313528
chrX SLC25A5 119469779 A C 0.204 ENSP00000360671.4:p.Gly73Ser rs134313528
chrX SLC25A5 119469784 A T 0.184 ENSP00000360671.4:p.Ile79Phe rs14128607
| chr | SLC25A5 | 119470481 | G   | C  | 0.444 | ENSP00000360671.4:p.Arg236Pro | rs114413582 |
|-----|---------|------------|-----|----|-------|-------------------------------|------------|


### Table S3. Mutations found in the WES for chromosome/telomere instability and the VEGF-angiogenesis pathway in sporadic CAS sequenced individuals (non-POT1 mutation carriers).

| Pathway | Sample | Gene | Genomic position | Reference allele | Alternative allele | ALT Allelic fraction | Amino acid change | dbsnp | MAF |
|---------|--------|------|------------------|------------------|-------------------|---------------------|-------------------|-------|-----|
| **NT1*** | TP53BP2 | 223991119 | G | T | 0.213 | Q229K | rs34683843 | 0.0467 |
| **NT2*** | TP53BP1 | 43762077 | TGGGATAGG | TGG | 0.205 | PI454- | - | - |
| ATM | 142215368 | GGAAGTAA | - | - | 0.215 | - | - | - |
| **NT3*** | TP53 | 7578503 | C | T | 0.451 | V143M | - | - |
| **T1** | TP53BP1 | 43762077 | TGGGATAGG | TGG | 0.205 | PI454- | - | - |
| ATM | 142215368 | GGAAGTAA | - | - | 0.215 | - | - | - |
| **T2** | BRCA1 | 41245120 | T | A | 0.128 | N810Y | rs28897682 | 0.0279 |
| ATM | 108200982 | T | A | A | 0.166 | H1488Q | - | - |
| **T3** | ATM | 108165638 | TTTAGGAAT | CTCT | 0.423 | -1593 | - | - |
| **T4** | BRCA1 | 41245120 | T | A | 0.128 | N810Y | rs28897682 | 0.0279 |
| | CDK8 | 26911760 | C | T | 0.310 | S62L | - | - |
| TP53 | 7578503 | C | T | 0.376 | V143M | - | - |
| TP53 RP2 | 223991119 | G | T | 0.213 | Q229K | rs34683843 | 0.0467 |
| ATM | 108200982 | T | A | A | 0.166 | H1488Q | - | - |
| **NT2***; **T3** | ATM | 108175462 | G | A | 0.202 | D1853N | rs1801516 | 0.0788 |
| Mutations involved in Angiogenesis | Gene | Position | Allele 1 | Allele 2 | Allele Frequency | SNP ID 1 | SNP Frequency 1 | SNP ID 2 | SNP Frequency 2 |
|-----------------------------------|------|----------|----------|----------|-----------------|---------|----------------|---------|----------------|
| NT1**                            | KDR  | 55979558 | C        | T        | 0.184           | V297I   | rs2305948      | 0.1310  |
|                                   | RASA2| 141326602| T        | C        | 0.422           | rs295322| 0.1140         |         |
|                                   | DOCK1| 128810642| C        | T        | 0.273           | rs9418773| 0.1230         |         |
|                                   | RICTOR| 38952300 | G        | A        | 0.179           | S1042L  | rs200988874    | 0.0005  |
|                                   | IQGAP1| 90996087 | G        | A        | 0.189           | E415K   | rs295322       | 0.1140  |
|                                   | SRC  | 36012590 | A        | G        | 0.304           | S12G    |               |         |
|                                   | PIK3C2G| 18691253 | C        | T        | 0.435           | rs12315010| 0.0389         |         |
|                                   | NRAS | 115256530| G        | T        | 0.134           | Q61K    | rs121913254    |         |
|                                   | PDK1 | 2607915  | CT       | C        | 0.310           |         |               |         |
| NT2*                             | RASA4| 102246366| G        | A        | 0.124           | R123W   | rs139960113    | 0.0020  |
|                                   | SHOX2| 157823580| TACA     | T        | 0.556           | p.77_78del|               |         |
| NT3*                             | NRG2 | 139231255| C        | T        | 0.345           | R577H   | rs75431091     | 0.0229  |
| T1                               | SPTAN1| 131367372| A        | G        | 0.204           | N1260S  |               |         |
|                                  | FGF22| 640065   | C        | T        | 0.256           | S47F    |               |         |
| T2                               | PIK3C2G| 18443809 | C        | A        | 0.107           | A261E   | rs7133666      | 0.0417  |
|                                  | RASA2| 141299289| G        | A        | 0.172           | S557N   |               |         |
|                                  | MAP2K1| 66736993 | G        | T        | 0.243           |         |               |         |
|                                  | IQGAP1| 91025227 | T        | G        | 0.435           | L1122R  |               |         |
|                                  | VAV3 | 108145045| C        | T        | 0.172           | E732K   |               |         |
|                                  | DUSP8| 1579018  | T        | C        | 0.234           |         |               |         |
| T3                               | PIK3C2G| 18473929 | C        | T        | 0.432           | Q391*   |               |         |
|                                  | RASA2| 141328901| G        | A        | 0.134           | G839R   | rs2228246      | 0.0952  |
|                                  | PLCG1| 39792063 | A        | G        | 0.345           | S279G   |               |         |
|                                  | RICTOR| 38960053 | C        | T        | 0.180           | V627I   |               |         |
|                                  | DUSP8| 1579306  | A        | G        | 0.172           |         |               |         |
| T4                               | RASA4| 102235804| G        | A        | 0.102           | T340I   |               |         |
|                                  | RASA4| 102246393| C        | T        | 0.165           | E114K   |               |         |
|                                  | ITGAV| 187540655| G        | A        | 0.184           |         |               |         |
|                                  | PTPRQ| 81004265 | A        | C        | 0.436           | E1589D  | rs201569993    | 0.0006  |
|                                  | PIK3C2G| 18656259 | C        | T        | 0.174           | H1021Y  |               |         |
| Gene   | Chromosome | Change | Base 1 | Base 2 | Frequency | Mutation |
|--------|------------|--------|--------|--------|-----------|----------|
| PRKCQ  | 6498721    | G      | A      | 0.102  | A521V     |
| SPTANI | 131377915  | G      | A      | 0.304  | R1718H    |
| MAP2K1 | 66777404   | C      | T      | 0.174  | A257V     |
| VEGFA  | 43738526   | C      | G      | 0.184  | A28G      |

* Confirmed constitutional ** Confirmed somatic.
Table S4. Actionable variants found in the sequencing experiment with IBM Watson for Genomics platform for T1 patient.

| Gene  | Actionable variant | Variant evidence (Pubmed) | FDA-approved drug for Angiosarcoma | Other molecules with clinical evidence (NCT) |
|-------|--------------------|---------------------------|-----------------------------------|-------------------------------------------|
| *MSH6* | p.Glu1088fs        | 12019211                  | Pembrolizumab                     | Nivolumab (NCT0366819)                   |
| *TP53*  | p.Arg175His        | 8510927; 17401432         | -                                 | Adavosertib (NCT01827384)               |
|       |                    |                           |                                    | Transferrin (NCT02354547)               |
| *ARID1A* | p.Pro21del      | 23097632                  | -                                 | **                                        |
| *ATR*   | p.Ile774fs        | 22960745                  | -                                 | **                                        |
| *NOTCH* | p.Pro2415del      | 15472975*; 25564152*      | -                                 | **                                        |

NCT: Number of Clinical Trial

* Similar variant within the same domain.

** Compelling preclinical evidence and/or case study reports support the biomarker as being predictive of response to this drug
Table S5. Actionable variants found in the sequencing experiment with IBM Watson for Genomics platform for T4 patient.

| Gene | Actionable variant | Variant evidence (Pubmed) | FDA-approved drug for Angiosarcoma | Other molecules with clinical evidence (NCT) |
|------|-------------------|---------------------------|-----------------------------------|---------------------------------------------|
| CDK2A | p.Arg99Pro        | 20340136; 19260062         | -                                 | Palbociclib (NCT03239015)                  |
| TP53  | p.Val143Met       | 23469205                  | -                                 | Adavosertib (NCT01827384)                 |
|       |                   |                           |                                    | Transferrin (NCT02354547)                 |
| KIT   | Amplification     | 21523721; 17189383; 16166280 | Pazopanib (NCT01462630)*          | Pazopanib (NCT03628131)                  |
|       |                   |                           |                                    | Imatinib (NCT02461849)                    |
|       |                   |                           |                                    | Nilotinib (NC02379416)                   |

NCT: Number of Clinical Trial
* Phase II study