

# Clinical and functional analysis of the germline *TP53* p.K164E acetylation site variant

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**Abstract** *TP53* plays a critical role as a tumor suppressor by controlling cell cycle progression, DNA repair, and apoptosis. Post-translational modifications such as acetylation of specific lysine residues in the DNA binding and carboxy-terminus regulatory domains modulate its tumor suppressor activities. In this study, we addressed the functional consequences of the germline *TP53* p.K164E (NM\_000546.5: c.490A>G) variant identified in a patient with early-onset breast cancer and a significant family history of cancer. K164 is a conserved residue located in the L2 loop of the p53 DNA binding domain that is post-translationally modified by acetylation. *In silico*, *in vitro*, and *in vivo* analyses demonstrated that the glutamate substitution at K164 marginally destabilizes the p53 protein structure but significantly impairs sequence-specific DNA binding, transactivation, and tumor cell growth inhibition. Although p.K164E is currently considered a variant of unknown significance by different clinical genetic testing laboratories, the clinical and laboratory-based findings presented here provide strong evidence to reclassify *TP53* p.K164E as a likely pathogenic variant.

[Supplemental material is available for this article.]

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## INTRODUCTION

Clinical laboratories offer a variety of diagnostic services that include sequencing of single genes, gene panels, exomes, genomes, and transcriptomes. The complexity of genetic testing goes along with the critical need to classify variants according to their pathogenicity (Richards et al. 2015). The criteria to evaluate the pathogenicity of genetic variants include but are not limited to the prevalence in affected individuals, segregation analysis, associated cancer phenotypes, functional studies, allele frequency from population databases, and type of mutation and its predicted effect, among others (Duzkale et al. 2013; Richards et al. 2015). However, healthcare providers are often challenged by the discovery of variants that cannot be categorized into strict and clear classifications, referred to as variants of uncertain significance (VUSs) (Federici and Soddu 2020).

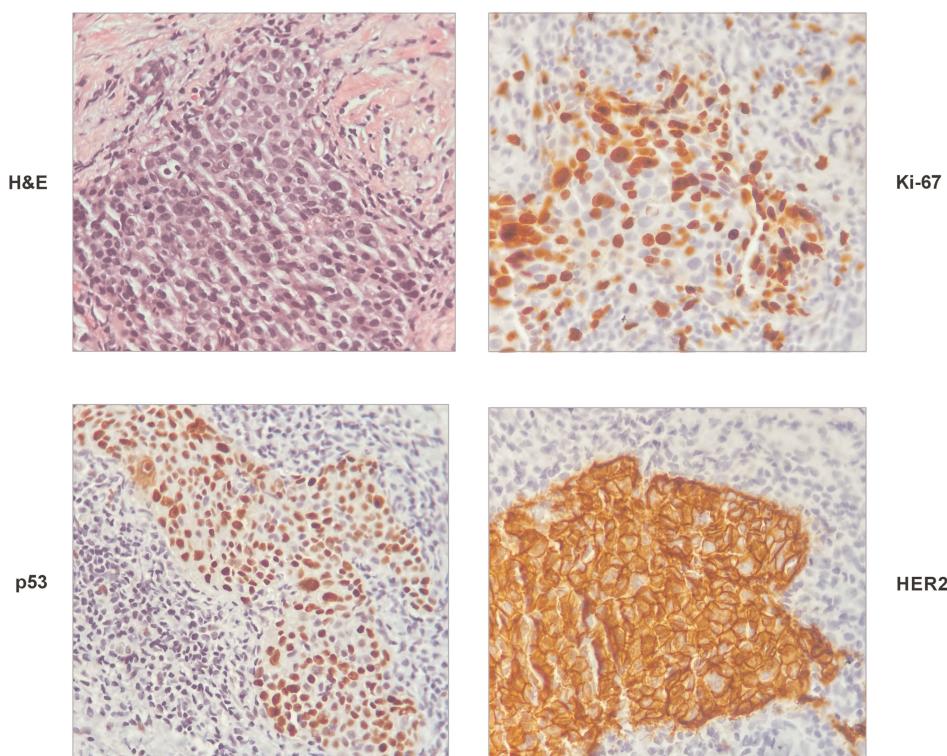
Most variants that are classified as VUSs are not clinically actionable for identifying individuals at risk or for driving treatment; however, there are cases that warrant further investigation for the benefit of the patients. Here, we present a proband with early-onset breast cancer with the germline *TP53* p.K164E (NM\_000546.5: c.490A>G) variant, classified as a VUS by different clinical genetic testing laboratories, that segregates in a family with high cancer risk. The K164 residue is reportedly an important site that is post-translationally

modified by acetylation, which plays a role in the activation of p53 as a tumor suppressor (Gu and Roeder 1997; Tang et al. 2008). Here, we provide clinical and experimental evidence to consider reclassification of the *TP53* p.K164E as a likely pathogenic variant, which could help inform the clinical management of carriers and their at-risk relatives.

## RESULTS

### Proband Clinical Presentation

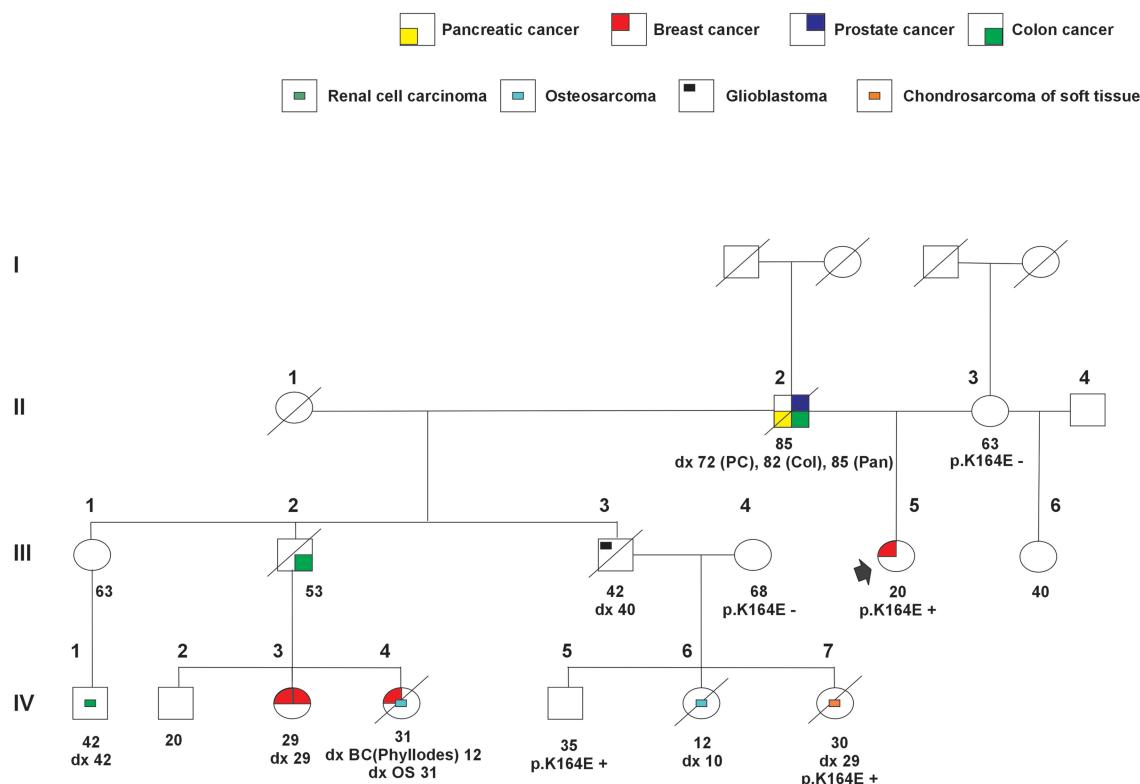
The patient is a 20-yr-old female who was diagnosed with invasive breast carcinoma. Ultrasound imaging detected an  $18.5 \times 13.4$ -mm mass located in the upper outer quadrant of her left breast with no lymph node invasion. Histopathological analysis of a biopsy sample confirmed the diagnosis and demonstrated that the tumor was negative for the estrogen receptor (EP1 clone) and progesterone receptor (PgR636 clone) but positive for c-erbB-2 (HER2; score 3+). In addition, tumor cells showed strong nuclear immunoreactivity for p53, and ~70% were positive for the nuclear antigen Ki-67 (Fig. 1). Immunostaining also demonstrated that the tumor was negative for cytokeratin 5/6 (D5/16 B4 clone) and positive for epithelial cytokeratin (AE1/AE3 clone) (data not shown), confirming the epithelial origin for this HER2-enriched breast cancer (Goldhirsch et al. 2013). The proband received neoadjuvant chemotherapy with four cycles of doxorubicin  $60 \text{ mg/m}^2$  and cyclophosphamide  $600 \text{ mg/m}^2$  followed by paclitaxel  $80 \text{ mg/m}^2$  for 12 wk and  $6 \text{ mg/kg}$  trastuzumab every 3 wk for 1 yr. A unilateral mastectomy was performed upon completion of chemotherapy.



**Figure 1.** Histopathological analysis. Invasive breast carcinoma positive for the p.K164E variant was stained by hematoxylin and eosin (H&E). Tumor cells express high levels of p53, Ki-67, and HER2.

### Genomic Analyses and Family History of Cancer

Germline DNA of the proband was analyzed using a panel of 45 cancer predisposition genes revealing only the heterozygous *TP53* p.K164E (NM\_000546.5: c.490A>G) variant. The family history of cancer includes her father (II-2), who developed prostate cancer at age 72, colon cancer at age 82, and pancreatic cancer at age 85. The proband's mother (II-3) is alive and well at age 63 (Fig. 2). The proband has three half-siblings on the paternal side: a half-sister (III-1) who is alive and well at age 63, with a son (IV-1) who developed renal cell carcinoma at age 42; a half-brother (III-2) who died from colon cancer at age 53, whose youngest daughter (IV-4) was diagnosed with a malignant phyllodes tumor of the breast at age 12 and died from osteosarcoma (vertebral column) at age 31 and whose older daughter (IV-3) developed bilateral breast cancer at age 29; and another half-brother (III-3) who was diagnosed (40 yr old) with and later died (age 42) from glioblastoma. None of the half-siblings underwent genetic screening for cancer predisposition genes. However, the half-brother (III-3) had three children, two of whom died from cancer: a daughter diagnosed with osteosarcoma at age 10 (IV-6) and a younger daughter diagnosed with chondrosarcoma of soft tissue (right iliac) at age 29 (IV-7) who was positive for the *TP53* p.K164E variant both in the germline and tumor. Subsequently, his son (IV-5), who is currently healthy at age 35, was also found to harbor the *TP53* p.K164E variant based on a panel of 94 cancer predisposition genes. Although the proband (Fig. 2, III-5) does not clearly meet classical LFS criteria per se, the diagnosis of classic LFS for the family was established in the daughter of the proband paternal half-brother (Fig. 2, IV-7).

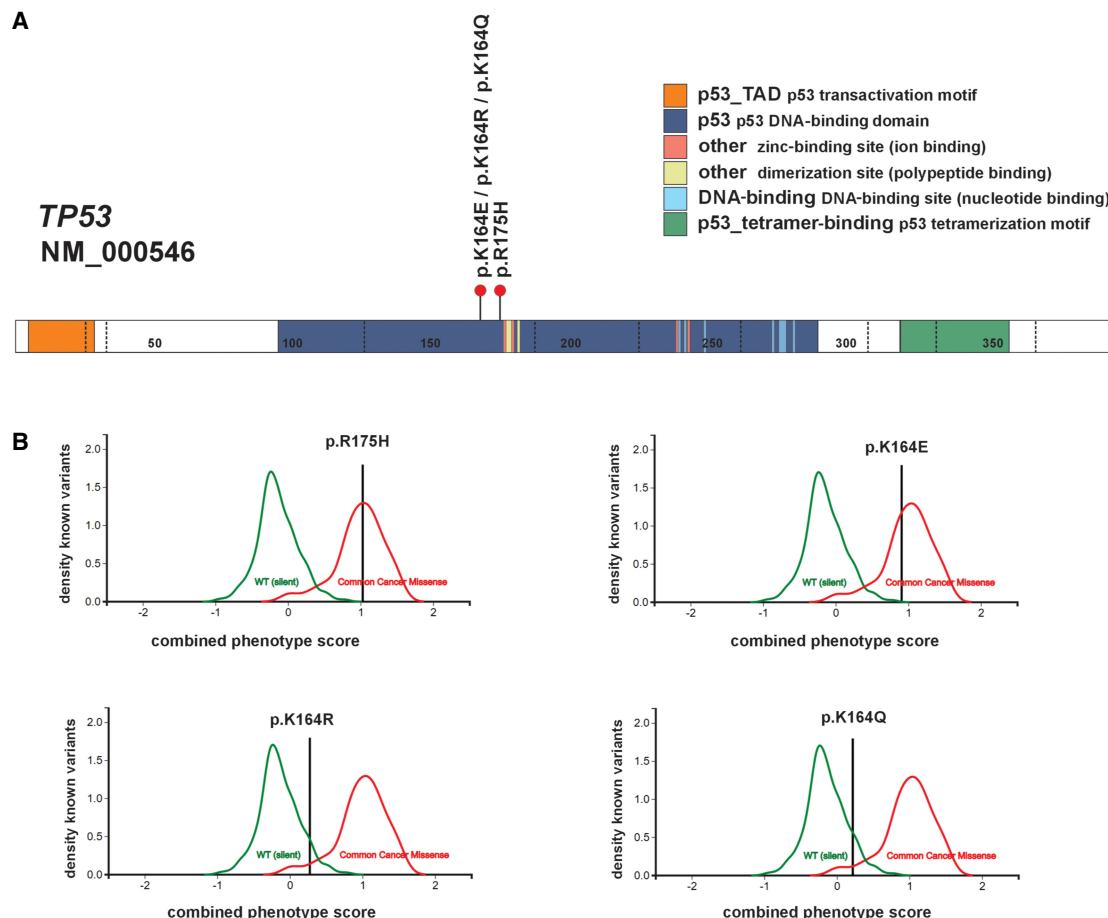


**Figure 2.** Pedigree of proband with strong family history of cancer. Proband with germline *TP53* p.K164E variant diagnosed with invasive breast carcinoma at age 20 and additional family members presenting with cancer and age of diagnosis. Individuals tested for p.K164E variant are indicated.

### TP53 p.K164E Variant Interpretation

The TP53 p.K164E germline variant was recently reported in a Chinese breast cancer study in a woman with early-onset breast carcinoma (24 yr old), negative for *BRCA* mutations and a significant family history of cancer (Kwong et al. 2020). Although TP53 p.K164E has not been previously reported in public databases as a germline mutation, it has been listed as a somatic variant in the COSMIC database ( $n = 32$ ; variant ID: COSV52728094), The TP53 Database ( $n = 25$ ), cBioPortal for Cancer Genomics ( $n = 4$ ), and Cancer Hotspots ( $n = 9$ ) in association with multiple tumor types including biliary tract, central nervous system, breast, ovarian, liver, lung, head and neck, and leukemia. The TP53 p.K164E variant is not found in the Flossies and Global Biobank Engine databases; however, it was documented in a single male individual in the recently released gnomAD (v4.0.0) with an allele frequency of 0.000001590.

TP53 p.K164E (c.490A>G; rs879254249) (Fig. 3A) is currently classified as a VUS by ClinVar ( $n = 4$ ; variation ID: 246416). However, multiple software tools (PolyPhen-2 = damaging; SIFT = damaging; Align GVGD = C55; and CADD = 23.6) predict that the p.K164E variant is nonfunctional and potentially pathogenic. Furthermore, the phenotypic annotation of



**Figure 3.** Schematic of TP53 K164 variants and predicted functional phenotypes. (A) Schematic diagram of p53 and location of the p.K164 and LFS-associated p.R175H variants within the DNA binding domain. (B) Combined phenotype score as predicted by PHANTM for each variant.

TP53 mutations (PHANTM) classifier (<https://mutantp53.broadinstitute.org/>) calculated a combined phenotype score of  $0.904 \pm 0.286$  with a low level of transcriptional activity in yeast (14.5%), which is quite similar to the LFS-associated DNA binding mutant *TP53* p.R175H [combined phenotype score of  $1.025 \pm 0.388$ ; 10.11% wild-type (WT) transcription] (Fig. 3B).

### TP53 p.K164 Acetylation Site Variants

K164 is acetylated by CREB-binding protein (CBP) and p300, and this post-translational modification is thought to be involved in the activation of wild-type p53 (p53<sup>WT</sup>) in response to DNA damage (Tang et al. 2008). Naturally occurring germline variants at *TP53* codon 164 are rare, with only three related cases harboring the c.491\_494del alteration reported in The *TP53* Database. To further understand the functional consequence of the p.K164E variant and acetylation at this site, we compared the biochemical properties of p.K164E to (1) p.K164R (c.491A>G), which retains the positive charge, and (2) p.K164Q (c.490A>C), which serves as a mimetic for acetylation (Table 1; Li et al. 2002).

The *TP53* p.K164R (c.491A>G) variant was not observed in the Flossies and Global Biobank Engine databases but was observed in two individuals in the updated gnomAD v.4.0.0, with an allele frequency of 0.00002192. This variant is not annotated in ClinVar but was reported as a somatic mutation in The *TP53* Database ( $n = 2$ ), as well as the cBioPortal ( $n = 1$ ) and COSMIC ( $n = 1$ ; variant ID 45428) databases. Furthermore, the *TP53* p.K164Q (c.490A>C) variant was also not found in the general population or listed in ClinVar but was included as a somatic variant in The *TP53* Database ( $n = 5$ ) and Cosmic database (variant ID 44521;  $n = 2$ ) in association with diverse tumor types including breast, lung, soft tissue, and testis. The PHANTM classifier predicts that the *TP53* p.K164R and p.K164Q variants retain substantial WT activity (Fig. 3B).

**Table 1.** *TP53* p.K164 acetylation site variants

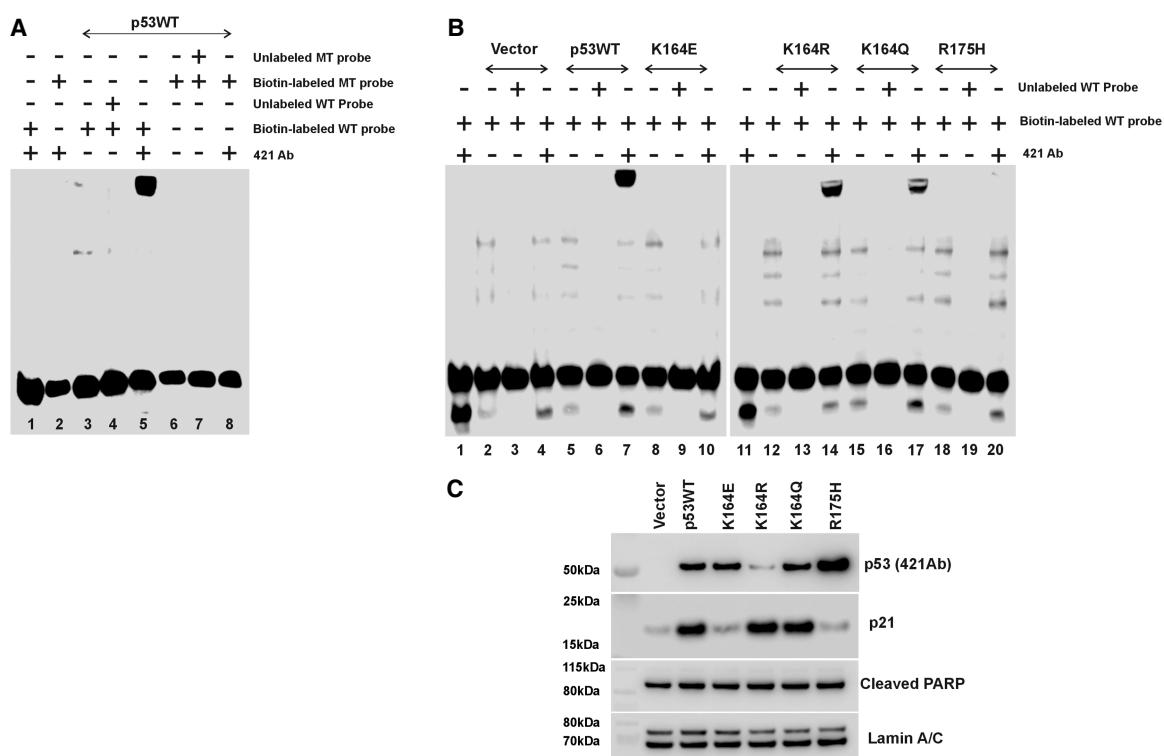
Gene Chr	Variant	HGVS DNA ref.	HGVS protein ref.	Variant type	Predicted effect	dbSNP/ dbVar ID	Genotype	ClinVar ID	Parent of origin	Observed effect
TP53 Chr 17	p.K164E c.490A>G	NM 000546.5	UniProt PO4637	Missense Substitution	rs879254249	Heterozygous	246416	Father	Impairs sequence-specific DNA binding, p53 transcriptional activity, and tumor cell growth inhibition	
TP53 Chr 17	p.K164R c.491A>G	NM 000546.5	UniProt PO4637	Missense Substitution	Not reported	Not reported			Functional in transactivation and colony growth suppression	
TP53 Chr 17	p.K164Q c.490A>C	NM 000546.5	UniProt PO4637	Missense Substitution	Not reported	Not reported			Functional in transactivation and colony growth suppression	

## Predicted Stability of the TP53 K164 Variants

K164 residue lies within the L2 loop on the solvent-exposed surface of the DNA binding domain of p53 and does not directly contact DNA based on available structural data (PDB 1 TUP; Supplemental Fig. 1). Analysis by FoldX (Schymkowitz et al. 2005) shows that each of the K164 variants [glutamate (E), glutamine (Q), and arginine (R)] is predicted to modestly destabilize the p53 DNA binding domain by 1.3, 2.3, and 0.7 kcal/mol, respectively.

## TP53 p.K164E Is Defective in Sequence-Specific DNA Binding

Sequence-specific DNA binding was investigated using nuclear extracts prepared from Saos-2 cells transiently transfected with the CMV-only (negative control) vector and corresponding expression vectors for p53WT, p.R175H, and each of the K164 variants (K164E, K164R, and K164Q). Gel shifts were carried out using biotin-labeled probes corresponding to WT and mutant (MT) p53 DNA binding consensus sites in the human p21<sup>CIP1</sup> promoter (see Methods). p53WT specifically bound the WT DNA probe (lane 5), but not the MT probe (lane 8) upon the addition of PAb421, as shown in Figure 4A (Gu and Roeder 1997). Similarly,



**Figure 4.** The p.K164E variant is defective in sequence-specific DNA binding. p53WT and K164 variant proteins were prepared from transiently transfected Saos-2 cells and analyzed for DNA sequence-specific binding by electrophoretic mobility shift assay (EMSA) as described in the Methods. (A) WT protein selectively bound the p53WT DNA consensus site probe and was super-shifted by the p53 monoclonal antibody PAb421 (lane 5) but not the mutant p53 consensus site (lane 8). (B) K164E and R175H failed to bind the p53WT consensus site probe (lanes 10 and 20, respectively), whereas K164R (lane 14) and K164Q (lane 17) were competent for binding. (C) Western blot analysis of nuclear extracts used in B demonstrates the level of expression of exogenous p53 and endogenous p21. Lamin A/C and Cleaved PARP serve as nuclear markers. A–C are representative of two independent experiments that have analyzed pools of four independently transfected cell cultures for each variant. (MT) Mutant, (WT) wild-type, (Ab) antibody.

p.K164R and p.K164Q also bound the WT probe in the presence of PAb421 (Fig. 4B, lanes 14 and 17, respectively). Although p.K164E and p.R175H were well-expressed (Fig. 4C), both proteins were defective in binding the WT DNA probe (Fig. 4B; lanes 10 and 20, respectively).

Collectively, these clinical, structural, and functional studies reveal that the *TP53* p.K164E variant is tightly linked with tumor formation and is significantly compromised in transactivation, target gene expression, and growth suppression. These findings provide strong evidence supporting the deleterious nature of the *TP53* p.K164E variant.

### Functional Analysis of K164 Variants

When ectopically expressed in p53-null Saos-2, *TP53* p.K164E failed to activate the expression of a promoter–luciferase reporter under the control of p53-responsive elements (Fig. 5A) or its endogenous target genes p21<sup>CIP1</sup> and MDM2 (Fig. 5B). Indeed, *TP53* p.K164E was as defective in transactivation as p.R175H, a common hotspot *TP53* mutant that is structurally misfolded (Bullock et al. 1997), functionally inactive (Kato et al. 2003), and highly associated with LFS core cancers (Fig. 5A,B; Olivier et al. 2010). Consistent with these results, *TP53* p.K164E and p.R175H were defective in suppressing the growth of Saos-2 cells in colony reduction assays (Fig. 5C,D). In contrast, p53WT and the p.K164R and p.K164Q variants were functional in transactivation (promoter–reporter; endogenous p21<sup>CIP1</sup> and MDM2) and colony growth suppression (Fig. 5A–D).

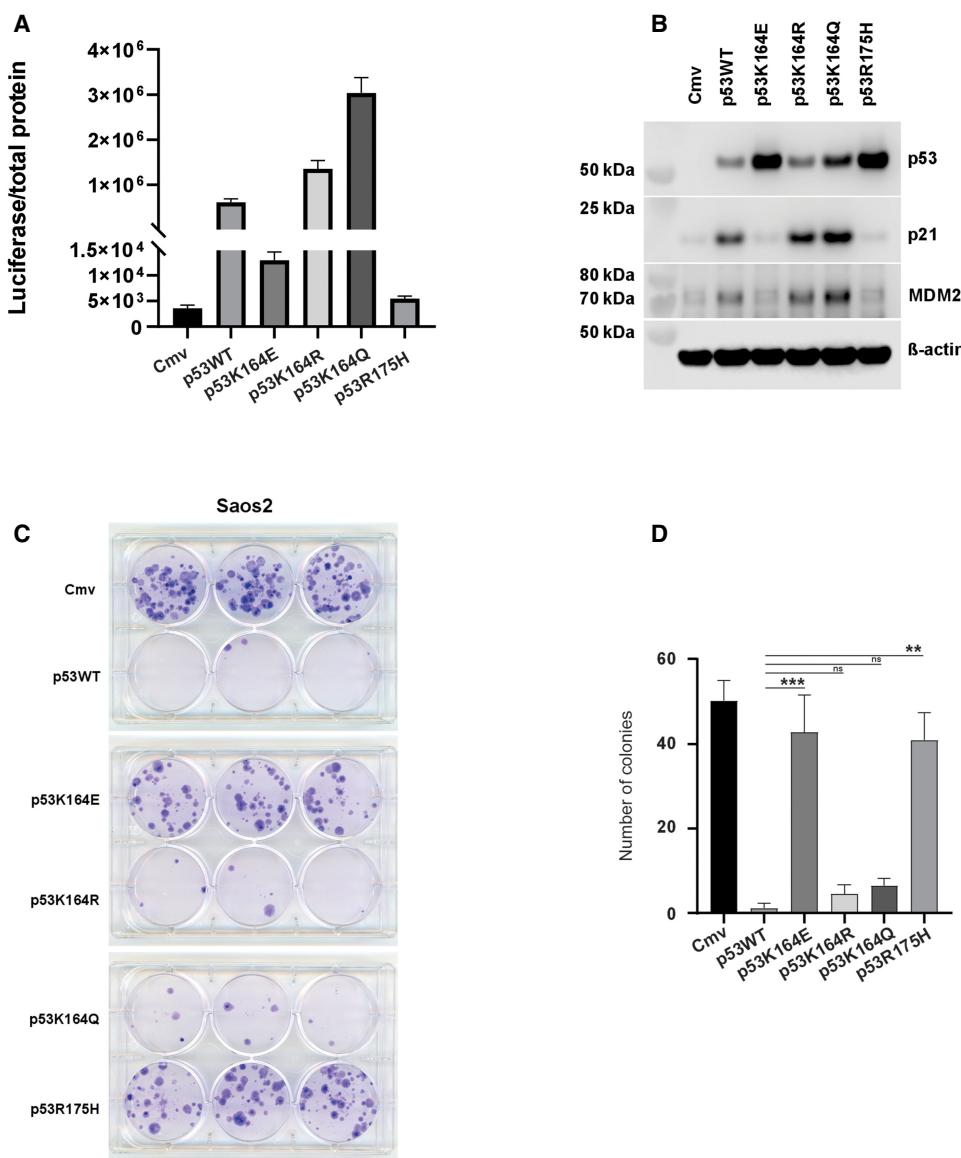
## DISCUSSION

Here, we report the identification of a rare germline *TP53* p.K164E mutation in a young woman who developed invasive breast carcinoma and was negative for other cancer predisposition genes. The *TP53* p.K164E variant segregated in a family with a cancer history consistent with the clinical criteria for classic Li–Fraumeni syndrome (Malkin 2011). Functional studies demonstrated that p.K164E is significantly compromised in binding DNA in a sequence-specific manner, transactivating responsive promoter–reporter and endogenous target genes and suppressing the growth of tumor cells in colony reduction assays.

Approximately 70%–80% of families meeting classic LFS criteria are associated with pathogenic *TP53* variants (Strong 2003; Gonzalez et al. 2008). However, the concept of LFS is evolving because of the diversity of clinical presentation and associated germline *TP53* alterations that have varied impacts on p53 structure and function. Therefore, familial history of cancer should not be mandatory when considering *TP53* genetic testing (Frebourg et al. 2020).

p53 function is regulated by a multitude of covalent modifications, including phosphorylation, acetylation, ubiquitination, and methylation, among others (Bode and Dong 2004). Various acetyltransferases have been identified that modify p53 at lysines predominantly in the carboxyl terminus and central DNA binding domain (Sakaguchi et al. 1998). The acetylation of p53 is proposed to directly affect its transcriptional activity by opening its normally closed conformation, thereby enhancing its binding to specific response elements in target genes (Sakaguchi et al. 1998). Biochemical studies have demonstrated that K164 within the DNA binding domain is acetylated by CBP/p300, which positively contributes to its stability and function in regulating cell cycle arrest and/or apoptosis (Tang et al. 2008).

Substituting glutamate for K164 significantly compromised DNA binding, transcriptional regulation, and growth suppression roughly to the same degree as the *TP53* p.R175H DNA binding mutant often associated with LFS (Olivier et al. 2010). In contrast, the p.K164R substitution, which preserves the positive charge at this site but is unable to be acetylated, retained p53 activity, consistent with previous studies (Tang et al.



**Figure 5.** Functional activity of wild-type TP53 and p.K164 variants. p53WT, p53-R175H, and the K164 variants (K164E, K164R, and K164Q) were transiently transfected into p53-deficient Saos-2 cells. (A) Transcriptional activity was assessed by promoter-reporter assays. (B) Corresponding p53 protein expression was determined by Western blot analysis. (C,D) p53 colony reduction assays (C) and quantification of colony reduction assays conducted in triplicate (D). (\*\*)  $P < 0.01$ , (\*\*\*)  $P < 0.001$ , (n.s.) nonsignificant.

2008). We also demonstrated that the substitution of K164 with glutamine (p.K164Q), which mimics lysine acetylation, resulted in enhanced p53 transactivation of promoter-reporters and endogenous target genes, supporting the model that acetylation contributes to the activation of p53. Although the effects of the p.K164E, p.K164R, and p.K164Q variants on p53 stability were all predicted to be rather mild, substituting the negatively charged acidic amino acid (E) at this single site renders the p.K164E variant nonfunctional. The exact mechanism by which the glutamate substitution inactivates p53 remains to be determined.

The germline *TP53* p.K164E variant reported here was identified in a Brazilian proband with early-onset HER2<sup>+</sup> breast carcinoma, a molecular tumor subtype rare in *BRCA1/BRCA2* variant carriers (Tutt et al. 2021; Tomasello et al. 2022) but often associated with pathogenic germline *TP53* variants (Wilson et al. 2010; Silwal-Pandit et al. 2014; Fortuno et al. 2020; Evans et al. 2022). *TP53* p.K164E segregated with additional family members who developed sarcoma and brain cancer at young ages. A germline *TP53* p.K164E variant was also independently identified in a young woman with early-onset breast carcinoma with a rich family history of cancer from Southeast Asia (Kwong et al. 2020). Guidelines for classifying the pathogenicity of *TP53* variants are based on recommendations by the Clinical Genome Resource *TP53* Variant Curation Expert Panel (ClinGen *TP53* VCEP) (Fortuno et al. 2021). By applying current guidelines (version 1.4.0, released on July 5, 2023) the *TP53* p.K164E variant meets the following codes: PM2\_Supporting, PP1, PP3, PS3, and PS4\_Moderate, resulting in the classification of Likely Pathogenic. Collectively, these experimental, demographic, and clinical findings support the reclassification of the *TP53* p.K164E variant from VUS to Likely Pathogenic.

## METHODS

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### Ethics Approval

Written informed consent from patients and institutional review board approval, allowing genetic analysis of germline samples, were obtained by the primary physician in Brazil. Studies conducted at St. Jude Children's Research Hospital did not involve human subject research.

### Panel Genetic Testing

Genetic testing was conducted at Fleury Medicina e Saúde, São Paulo, Brazil. Genomic DNA from buccal swabs was enriched for targeted regions contained in the Panel Hereditary Cancer Fast Track using a hybridization-based protocol and sequenced using Illumina technology. The panel contains 45 cancer predisposition genes [APC (including promotor), ATM, BARD1, BLM, BRCA1, BRCA2, BRIP1, CDH1, CDK4, CDKN2A, CHEK2, EGFR, EPCAM, HRAS, KIT, MEN1, MET, MLH1 (including promotor), MRE11 (MRE11A), MSH2, MSH6, MUTYH, NBN, NF1, NF2, NTHL1, PALB2, PIK3CA, PMS1, PMS2, POLD1, POLE, PTEN (including promotor), RAD50, RAD51C, RAD51D, RB1, RECQL4, RET, SMAD4, SMARCA4, STK11, TP53 (including promotor), WT1, and XRCC2]. The variants were interpreted considering the patient's clinical condition and the classification protocol of variants from the American College of Medical Genetics (ACMG), with PM2, PP2, and PP3 criteria for the *TP53* p.K164E variant.

### Database Query: Population Database and Reported Variants

*TP53* p.K164E, p.K164R, and p.K164Q variants were interrogated in public genome sequence databases, including gnomAD (v4.0.0; >800,000 unrelated individuals as part of disease-specific and population genetic studies; <https://gnomad.broadinstitute.org>), Flossies (7000 European American and 3000 African American women older than age 70 who never had cancer; <https://whi.color.com/about>), and Global BioBank Engine (750,000 individuals including UK Biobank, Million Veterans Program and Biobank Japan; <https://biobankengine.stanford.edu>). In addition, the frequency of tumors harboring germline or somatic variants at these codons was determined based on those reported in The *TP53* Database (<https://tp53.isb-cgc.org>) and COSMIC (<https://cancer.sanger.ac.uk/cosmic>), cBioPortal (<https://www.cbioportal.org/>), and Cancer Hotspots (<https://www.cancerhotspots.org/>) databases.

### Structural Effects of Missense Variants at TP53 p.K164E

Structural effects of three amino acid substitutions at K164 [glutamic acid (Glu, E), arginine (Arg, R), and glutamine (Gln, Q)] within the DNA binding domain of the *TP53* (Wang et al. 2007) were predicted using FoldX (Schymkowitz et al. 2005) and visualized by PyMOL (<https://pymol.org/2/>).

### Cell Lines

The human osteosarcoma cell line Saos-2 (*TP53*-null) was maintained as monolayers in Dulbecco's modified Eagle's medium with D-glucose (ThermoFisher Scientific) supplemented with 10% fetal bovine serum, MEM nonessential amino acids solution (Thermo Fisher Scientific), 2.5 mmol/L-glutamine, and penicillin-streptomycin (complete media) at 37°C under 5% CO<sub>2</sub>. Saos-2 cells were authenticated by STR profiling (Promega) (Supplemental Table 1) and demonstrated to be mycoplasma-free using the Venor GeM mycoplasma detection kit, PCR-based (Sigma).

### Expression Constructs

The p.K164E, p.K164R, and p.K164Q mutants were constructed using QuikChange II site-directed mutagenesis kit (Stratagene) with the forward primer 5'-GCCATGGCCATCTACGA GCAGTCACAGCACA-3' and reverse primer 5'-TGTGCTGTGACTGCTCGTAGATGGCC ATGGC-3' for the p.K164E variant, forward primer 5'-GCGCCATGGCCATCTACAGGCAG TCACAG-3' and reverse primer 5'-CTGTGACTGCCTGTAGATGGCCATGGCGC-3' for the p.K164R variant, and forward primer 5'-GCCATGGCCATCTACCAGCAGTCACAGCACA-3' and reverse primer 5'-TGTGCTGTGACTGCTGGTAGATGGCCATGGC-3' for the p.K164Q variant. The pCMV-Neo-Bam expression vector containing wild-type *TP53* cDNA (arginine at codon 72) served as the template. These plasmids were provided by B. Vogelstein, Johns Hopkins Oncology Center. The entire coding sequence of each plasmid was confirmed by direct DNA sequencing.

### Luciferase Assays

Saos-2 cells were transiently cotransfected with 100 ng of the p53-responsive luciferase reporter PG13-Luc (Addgene) that contains 13 copies of the p53 DNA binding consensus site upstream of the polyoma promoter and 5.5 µg of pCMV-Neo-Bam (vector only) or 5.5 µg of pCMV-Neo-Bam expressing WT p53, p.R175H, p.K164E, p.K164R, or p.K164Q using Lipofectamine LTX (Thermo Fisher Scientific), as previously described (Pinto et al. 2020, 2022). Cells were harvested at 48 h after transfection and lysed in passive lysis buffer (Promega) containing protease inhibitor cocktail and halt phosphatase inhibitor cocktail (Thermo Fisher Scientific). Protein yield was determined by the Bradford method (Bio-Rad Laboratories). The relative luciferase activity was determined using the single luciferase assay system (Promega) as per the manufacturer's directions by adding 20 µL of protein lysate to 100 µL of luciferase assay reagent (Promega), followed by a 2-sec measurement delay and then 10-sec measurement read in an Optocomp I luminometer (MGM Instruments). Relative light units (RLUs) were normalized to total protein (Pinto et al. 2020, 2022).

In parallel, total protein extracts (13 µg) were separated on NuPAGE 4%–12% Bis-Tris gels (Thermo Fisher Scientific) and transferred to nitrocellulose membranes as previously reported (Pinto et al. 2020, 2022). Membranes were incubated with mouse monoclonal anti-human p53 DO-1 antibody (1:500; EMD Millipore), rabbit p21 Waf1/Cip1 (1:1000; Cell Signaling), mouse anti-mdm2 antibody (1:2500; Abcam), and mouse monoclonal β-Actin antibody (1:4000; Sigma-Aldrich), followed by corresponding secondary antibodies. Proteins were detected using a LI-COR Odyssey infrared scanner.

### Colony Reduction

Cells were transfected with p53 expression vectors using Lipofectamine and selected in a complete medium containing 800 µg/mL G418 antibiotic (Invitrogen) for up to 21 d with the change of media every 3 d. Cells were washed with Dulbecco's phosphate-buffered saline (D-PBS), fixed with 100% methanol, stained with 1:20 Giemsa (diluted in D-PBS) stain for 45 min, washed briefly, and air-dried before visualization and image capture (Epson perfection V600).

### Electrophoretic Mobility Shift Assay

Biotinylated oligonucleotides corresponding to the p53-responsive elements in the p21 promoter were prepared as previously described (Kendig et al. 2017). The oligonucleotides (5'-CATCAGGAACATGTCCCAAACATGTTGGCGTCGGCTGTCGGAGGAACATGTCCCAAACATGTTGAGCTCT-3') containing two repeats of the p53 consensus sequence (underlined) in the human p21<sup>CIP1</sup> promoter and its antisense strand DNA were synthesized and labeled with Biotin 3' end DNA labeling kit (WT probe) (Thermo Scientific). To determine binding specificity, probes with a mutant binding site (5'-CATCAGAAAATTTCCCAAATTTGAGCTCT-3') were included as a negative control (MT probe), and unlabeled WT binding site oligonucleotides were used in competition assays.

Nuclear extracts were prepared from Saos-2 cells transiently transfected with p53WT and expression constructs (K164E, K164R, K164Q, and R175H) using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher). Lamin A/C (1:2000, Cell Signaling) and Cleaved PARP (1:1000; Cell Signaling) were analyzed by Western blot as nuclear marker loading controls. Nuclear extracts (2 µL of NE-PER reagent extract = 1.7 µg/µL) were incubated in 1× binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT at pH 7.5), 2.5% glycerol, 5 mM MgCl<sub>2</sub>, 50 ng/µL Poly (dI.dC), 0.05% NP-40, 4 pmol of unlabeled DNA, and 20 fmol of biotin-labeled probes (WT and MT) in a total volume of 20 µL. Super-shift reactions were performed using nuclear extracts, biotin-labeled DNA probe, and anti-p53 monoclonal antibody PAb421 (OP03, Calbiochem). Competition experiments were carried out by adding 200× unlabeled DNA to the supershift assay. The protein–DNA complex was separated from unbound probe using a precast DNA retardation gel (6%; Thermo Fisher) for 2 h at 4°C in a 0.5× TBE running buffer. The gel was then transferred to a positively charged Biodyne B nylon membrane (Thermo Fisher). Membranes were cross-linked, blocked in 16 mL of blocking buffer (Thermo Fisher), incubated in a solution containing 1:300 dilution of stabilized Streptavidin–horseradish peroxidase conjugate, and visualized using a chemiluminescent nucleic acid detection module (Thermo Fisher).

## ADDITIONAL INFORMATION

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### Database Deposition and Access

The sequencing data were generated as part of clinical testing, so the underlying raw data are not consented for deposition to a public database. The variant has been deposited in ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) and can be found under accession number VCV000246416.6.

### Ethics Statement

The study was reviewed and approved by the Brazilian Research Ethics Committee (CEP/CONEP approval number: 3.342.297). All participants were de-identified and provided their written informed consent to participate in this study.

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### Author Contributions

E.M.S.F.R. performed the clinical description of the case. E.M.P., J.W., A.H.P., R.W.K., and G.P.Z. participated in the design and execution of the experiments and data analysis. All authors contributed to the preparation of the manuscript and figures and have approved the final version.

### Competing Interest Statement

The authors have declared no competing interest.

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