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Title page

Overexpression and promoter mutation of the *TERT* gene in malignant pleural mesothelioma

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Running Title

***TERT* promoter mutation in mesothelioma**

Abstract and keywords

Abstract

Malignant pleural mesothelioma (MPM) is a very aggressive tumor with no known curative treatment. Better knowledge of the molecular mechanisms of mesothelial carcinogenesis is required to develop new therapeutic strategies. MPM, like all cancer cells, need to maintain telomere length to prevent senescence. Previous studies suggested that the telomere lengthening mechanism in MPM is based mainly on telomerase activity. For this reason, we focused on the key catalytic enzyme, *TERT* (telomerase reverse transcriptase), by analyzing its gene expression in MPM and by studying the mechanism underlying its up-regulation. We used our large collection of MPM composed of 61 MPM in culture and 71 frozen MPM tumor samples. Evaluation of *TERT* mRNA expression by quantitative RT-PCR showed overexpression in MPM in culture compared to normal mesothelial cells, and in MPM tumor samples compared to normal pleura. We identified a “hot spot” of mutations in the *TERT* gene core promoter in both MPM in culture and in MPM tumor samples with an overall frequency of 15%. Furthermore, data clearly identified mutation in the *TERT* promoter as a mechanism of *TERT* mRNA up-regulation in MPM. In contrast, gene copy number amplification was not associated with *TERT* overexpression. Then, we analyzed the clinicopathological, etiological and genetic characteristics of MPM with mutations in the *TERT* promoter. *TERT* promoter mutations were more frequent in MPM with sarcomatoid histologic subtype ($P<0.01$), and they were frequently associated with *CDKN2A* gene inactivation ($P=0.03$). In conclusion, a subgroup of MPM presents *TERT* promoter mutations, which lead to *TERT* mRNA up-regulation. This is the first recurrent gain-of-function oncogenic mutations identified in MPM.

Keywords

Thoracic neoplasm; mesothelioma; asbestos; telomere; telomerase reverse transcriptase (TERT); mutation

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor arising in the pleural cavities. The major risk factor for MPM is past exposure to asbestos, a mineral fiber known as a carcinogenic xenobiotic, which induces genomic and genetic alterations¹. Other risk factors include exposure to non-asbestos fibers such as erionite, the effect of ionizing radiations, contamination by the Simian virus, SV40, and genetic factors such as germline mutations in the *BAP1* gene encoding a nuclear deubiquitinase^{2,3}. MPM is resistant to conventional anti-cancer therapies (surgery, radiotherapy and chemotherapy). Its aggressiveness and the lack of curative treatment are responsible for the poor prognosis associated with MPM. There is a strong need to increase our knowledge about the molecular alterations in MPM to develop new therapies.

Molecular aspects of mesothelial carcinogenesis include chromosomal abnormalities, gene mutations, epigenetic alterations, gene expression changes and, more generally, signal pathway dysregulation⁴. Concerning genetic alterations, mainly recurrent somatic mutations in tumor suppressor genes have been reported in MPM. The most frequently inactivated genes in MPM are *CDKN2A* and *CDKN2B* that reside at the same locus on chromosome 9 in 9p21 and encode cell cycle regulatory proteins, *NF2* that encodes a multifunctional protein linking cytoskeletal components with proteins in the cell membrane, and *BAP1*^{4,5}. Knowledge of gene mutations provides insight into specific signal pathways that are altered in MPM, opening the way for future therapeutic strategies based on molecular characteristics of tumors such as targeted therapies.

One of the hallmarks of cancer is the immortalization of the tumor cells that need to counteract telomeres that shorten at each round of DNA replication. In normal cells, the absence of a lengthening mechanism, telomeres become too short and tumor cell senescence occurs. The main telomere maintenance mechanism involves an enzyme complex, the telomerase, which synthesizes *de novo* telomeres. Another mechanism based on homologous recombination, the alternative lengthening of telomeres (ALT), has been described for maintaining telomere length⁶. However, while ALT mechanism was identified in 18% of peritoneal mesothelioma (44 cases)⁷, it was not found in an equivalent series of MPM (43 cases)⁸. Telomerase activity was detected between 91% and 100% of MPM, depending on the study^{8,9}. These results suggest that maintenance of telomere length in MPM relies mainly on telomerase activity and, consequently, on the expression of the key catalytic enzyme, TERT (telomerase reverse transcriptase). So far, only one study has analyzed TERT expression in MPM using formalin-fixed tumors by immunohistochemistry (68 cases) and *in situ*

hybridization (46 cases), and detected TERT expression in 99% of MPM, but mechanisms of TERT up-regulation are unknown¹⁰.

In this study, we evaluated *TERT* gene expression at the mRNA level in a large series of MPM, since a good correlation between telomerase activity and the level of *TERT* mRNA is generally observed in human cells¹¹. The recent evidence of mutations in the *TERT* promoter that may increase its transcription¹²⁻¹⁴ led us to determine the frequency of these mutations in MPM and their possible contribution to *TERT* overexpression. Correlation between *TERT* gene mutation and clinico-pathological, epidemiological and genetic characteristics of MPM was also examined.

Results and Discussion

***TERT* expression in MPM**

TERT gene expression was evaluated using qRT-PCR in 61 MPM in culture established in the laboratory, as reported in the literature^{15, 16}, and in 71 MPM frozen tumor samples obtained from the French mesobank network (biobanks of three French hospitals: CHU de Caen; CHRU de Lille; CHU de Nice) and the biobank collection of the Hôpital Européen Georges Pompidou (HEGP) in Paris. All MPM were diagnosed by the center for pathological diagnosis certification (MESOPATH French National group) that is responsible for MPM diagnosis certification in France¹⁷. As controls, normal mesothelial cells cultures (five cases) were established after scraping the visceral pleura from surgical resection of blebs from patients with spontaneous pneumothorax. Normal pleura (seventeen cases) were obtained after stripping and dissecting the parietal pleura from patients with lung cancer, mediastinal lymphoma, pulmonary emphysema or spontaneous pneumothorax. **Figure 1** shows significant higher expression of *TERT* mRNA in MPM in culture compared to normal mesothelial cells in culture (**Figure 1.a**), and in MPM tumor samples compared to normal pleura (**Figure 1.b**). In our series, 84% of MPM in culture and 85% of MPM tumor samples showed at least a two-fold increase in *TERT* gene expression compared to their respective controls. Our data are in agreement with the up-regulation of *TERT* protein in formalin-fixed MPM tumors, as previously described by immunohistochemistry in comparison to benign mesothelial lesions¹⁰, and demonstrate that *TERT* is overexpressed in MPM tumor cells at the transcriptional level.

Mutations in the *TERT* promoter

A recent study identified highly recurrent *TERT* promoter somatic mutations in human melanoma at two “hot spot” sites: g.1,295,228 and g.1,295,250, referred to as C228T and C250T, respectively (**Figure 2**)^{12, 13}. These somatic mutations were shown not to be restricted to melanoma and to be present in several types of tumors¹⁴. Transfection reporter gene assay in three human cell lines suggested that these mutations increase *TERT* promoter activity and may be involved in mRNA up-regulation¹³. Data concerning *TERT* mutation in MPM are very limited at this time. No mutation was found in four MPM tumor samples¹⁴. The *TERT* promoter was also studied in two MPM cell lines and mutation was found in the H2052 cell line¹³.

To clarify the presence or not of *TERT* promoter mutations in MPM, we performed DNA sequence analysis of the *TERT* promoter using our MPM sample collection. We identified *TERT* promoter mutations in 15.2% of MPM samples (20/132 cases), i.e., 19.7% of MPM in culture (12/61 cases) and

11.3% of MPM tumor samples (8/71 cases) (**Figure 2**). The lower *TERT* promoter mutation rate in MPM tumor samples than in MPM in culture could be related to decreased sensitivity to detect mutations in frozen tumors due to the presence of normal cells. Mutations were detected only in the C228T “hot spot” site, which is also the most frequently mutated in other cancers¹⁴. Sequence analysis showed that this mutation affected only one of the alleles of the *TERT* gene locus in MPM in culture. It is likely that this is a somatic mutation, as has been exclusively found in others tumors¹²⁻¹⁴. All mutations consist of a cytosine-to-thymine transition that generates a potential Ets/TCF binding site, as previously described^{12,13}. The presence of this transcription factor binding site could increase the *TERT* promoter activity and, consequently, *TERT* mRNA expression.

Role of *TERT* promoter mutation on gene expression

The relationship between *TERT* promoter mutation and *TERT* gene expression was then studied. Analysis of qRT-PCR data showed significantly higher levels of mRNA expression in MPM with *TERT* promoter mutation in both MPM in culture (**Figure 3.a**) and MPM tumor samples (**Figure 3.b**). Our results clearly identified genetic alterations of the *TERT* promoter as a mechanism of *TERT* mRNA up-regulation in MPM. However, it seems that mutation in the *TERT* promoter is not the only mechanism involved in *TERT* overexpression since several MPM without *TERT* promoter mutation showed a higher level of expression than normal tissue. It was suggested that up-regulation of *TERT* in cancer cells could result from an increase of the gene copy number¹⁸. The 5p chromosomal region, which encompasses the *TERT* gene locus, is a recurrent region of chromosomal alterations in MPM. Gain of this region is reported in 22% to 55% of MPM in recent studies using genomic high-throughput analyses⁴. In our collection of MPM in culture, the 5p15.3 region is gained in 49.5% of MPM (16/33 cases)¹⁶. Mutations in the *TERT* promoter and gain of the 5p15.3 region are not mutually exclusive since three mutated MPM showed a gain in the 5p15.3 region, while four mutated MPM showed no chromosomal alteration. Analysis of MPM without mutation in the *TERT* promoter showed no significant mRNA expression changes between MPM with or without gain in the 5p15.3 region (**Supplementary Figure S1**). Our results suggest that chromosomal amplification of the *TERT* gene is not involved in the up-regulation of *TERT* in MPM. A transcriptional mechanism could be responsible for *TERT* up-regulation in MPM without mutation in its promoter since *TERT* gene expression is regulated by several transcription factors or oncogenes, which may be dysregulated in MPM^{11, 19}. For example, MPM express c-Myc, a transcriptional activator of *TERT*²⁰. Epigenetic mechanisms, which regulated expression of several genes in MPM, could also be involved in *TERT* expression regulation, as it was shown in others tumor cell types^{4, 19}. Several mechanisms are

involved in *TERT* up-regulation in MPM, but it should be underlined that MPM with *TERT* promoter mutation have the highest gene expression of *TERT*.

Clinico-pathological, etiological and genetic characteristics of MPM with mutation in the *TERT* promoter

The main clinico-pathological characteristics and epidemiologic data of our MPM collection are summarized in [Supplementary Table S1](#). An association between mutation in the *TERT* promoter and histologic subtypes of MPM was observed. Histologic types were defined by the MESOPATH French National group, responsible for MPM diagnosis certification in France ¹⁷. Three main histologic subtypes are described for MPM: epithelioid, sarcomatoid and biphasic. Several unusual morphological variants have also been identified, including desmoplastic MPM, which are a sarcomatoid MPM with a predominance of dense collagenous stroma. The sarcomatoid, which have the worse prognostic compared to other histologic subtypes, and desmoplactic MPM (6/15 cases, 40.0%) showed a significantly higher frequency of mutation in the *TERT* promoter than epithelioid MPM (10/95 cases, 10.5%) ([Table 1](#)). Biphasic MPM, which consist of a combined epithelioid and sarcomatoid pattern, showed an intermediate rate of mutation (3/16 cases, 18.8%). A difference in the mutation rate between histologic subtypes was also observed for glioma, and the authors suggested that tumors with high frequencies ($\geq 15\%$) of *TERT* promoter mutations arose in tissues with relatively low rates of self-renewal ¹⁴. However, a low renewal rate of normal mesothelial cells in the pleura has been reported ²¹. One might suggest that MPM subtypes arise from mesothelial cells with different dividing properties, regarding their *in situ* heterogeneity and different capabilities in mesothelium regeneration ²².

No significant association was found between mutation in the *TERT* promoter and the other clinico-pathological characteristics of MPM ([Supplementary Table S1](#)). A trend can be observed in the epidemiological data. The evaluation of asbestos exposure in MPM patients was based on clinical reports or by interviewer-administered questionnaires. MPM patients were then broken down into three classes by an occupational hygienist based on the probability of exposure: ascertained, probable/possible and null. Higher frequency of mutation is observed in MPM of patients with ascertained asbestos exposure (15/75 cases, 20.0%) than in MPM of patients with probable/possible exposure (2/17 cases, 11.8%) or null exposure (2/29 cases, 6.9%), but these differences were not significant ($P=0.23$). A greater population would be needed to determine the relevance of these findings. The action mechanism of asbestos might facilitate escape from senescence in MPM. Asbestos is a well-known mutagen whose action mechanism involves oxidative stress and impairment of mitosis progression. Oxidative stress damages DNA and down-regulates the

expression of *TERT* mRNA²³. Prolonged mitosis was found to produce DNA damage at telomeres²⁴. *TERT* overexpression related to mutation in the *TERT* promoter could counteract the down-regulation and impact on telomere maintenance.

Genetic alterations in genes involved in mesothelial carcinogenesis (*CDKN2A*, *CDKN2B*, *BAP1*, *NF2*) and in more common cancer-related genes (*TP53*) were previously identified in our cell bank composed of 61 MPM in culture (published^{16, 25-27} and unpublished data). The genetic alteration profile of MPM in culture with *TERT* promoter mutation is shown in [Supplementary Figure S2 and Supplementary Table S2](#). *TERT* promoter mutations are found only in MPM with *CDKN2A* alteration. In comparison to the MPM without *TERT* promoter mutation, a significantly higher frequency of alterations in *CDKN2A* gene is observed in MPM with *TERT* promoter mutation ($P=0.03$) ([Table 1](#)). Genetic alterations in *NF2* and *CDKN2B*, which is the same locus as *CDKN2A*, are also more frequent than in MPM with no mutation in the *TERT* promoter. At the opposite, fewer mutations are found in *BAP1* gene, but these differences are not significant ([Table 1](#)). To analyze *CDKN2A* mutation on MPM tumor samples, we performed Multiplex Ligation-Dependent Probe Amplification (MLPA) for the 9p21 locus on 69/71 MPM tumor samples, as *CDKN2A* genetic alterations consisted mainly in exon deletions in MPM in culture (98% of overall mutation at the *CDKN2A* locus). The MLPA was carried out using SALSA ME024 kits (MRC-Holland, Amsterdam, The Netherlands) and peak height ratios lower than 0.7 were considered as indicative of deletion. The association between the alterations at the *TERT* promoter and *CDKN2A* gene were also observed in MPM tumor samples. Among the 8 MPM tumor samples mutated in *TERT* promoter, 6 showed exon deletion in the *CDKN2A* gene ([Supplementary Table S2](#)). By taken into account MPM in culture and MPM tumor samples, the genetic alterations in *CDKN2A* gene were more frequent in MPM with *TERT* promoter mutation ($P=0.01$). Data mining of the Horn *et al.* study showed also a higher frequency of *CDKN2A* alteration in melanoma with (57.3%) than without (31.4%) *TERT* promoter mutations ($P<0.01$)¹². *CDKN2A* plays a role in the regulation of senescence²⁸. Association of up-regulation of *TERT* by mutation and *CDKN2A* loss by inactivating mutation may reinforce an increase in the life span of MPM cells and their escape from senescence, one hallmark of neoplastic progression²⁹. It was also shown that P16/INK4A, one of the proteins encoded by the *CDKN2A* gene, repressed *TERT* expression at the transcriptional level in breast normal and cancer cells³⁰. It would be interesting to determine whether *TERT* promoter mutations still induce *TERT* overexpression in the presence of P16/INK4A in MPM.

The main finding of this study is the identification of *TERT* promoter mutation in MPM, which accounts for *TERT* overexpression. Interestingly, this mutation is associated to the MPM histologic phenotype and to genetic alterations in the *CDKN2A* tumor suppressor gene. To our knowledge, this

is the first “hot spot” oncogenic mutation identified in MPM. Only recurrent inactivating mutations in tumor suppressor genes have been identified in MPM as of this time ⁴. Identification of recurrent oncogenic mutation has a great impact on our knowledge of mesothelial carcinogenesis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Legends of figures

Figure 1. *TERT* gene expression in MPM.

TERT mRNA expression was analyzed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) in 61 MPM in culture (**a**) and 71 MPM frozen tumor samples (**b**). Five normal mesothelial cells in culture (**a**) and seventeen normal pleura samples were used as controls (**b**). Briefly, single-stranded cDNA was synthesized from 1.5 µg of Trizol-prepared total RNA using random primers and the high capacity cDNA reverse transcription kit (Life Technologies, Courtaboeuf, France). Real-time quantitative PCR was performed using pre-defined TaqMan probe and primer sets for *TERT* (Hs00972656_m1, Life Technologies) in an ABI PRISM® 7900HT System. Expression of *TERT* gene was normalized to internal control ribosomal 18S relative to the mean expression of the corresponding gene in normal mesothelial cells in culture, according to the $\Delta\Delta C_t$ method. Histograms are the mean \pm SEM. *P*-values determined by the Mann-Whitney test using GraphPad prism software (version 5.0b) are indicated above each dataset. Data show significant up-regulation of the *TERT* gene in MPM compared to normal mesothelial cells and tissues.

Figure 2. *TERT* promoter mutation.

Mutations in the *TERT* promoter were screened by Sanger sequencing in 61 MPM in culture and 71 MPM tumor samples. Briefly, the promoter region was amplified from genomic DNA by PCR, and fluorescent-based automated cycle sequencing was performed by the BigDye Terminator method (Life Technologies) using a multi-capillary sequencer (ABI 3130 Genetic Analyzer). Mutations were detected using Sequencher software (Gene Codes Corp., Ann Arbor, MI, USA) and verified by independent amplification and sequencing. Schematic representation of the *TERT* promoter is annotated with the localizations of the *TERT* transcription (TSS) and translation (ATG) start sites, the regions targeted by the specific primers used for the PCR amplification and sequencing (in green), and the two “hot spot” mutations (in red): g.1,295,228 and g.1,295,250, referred to as C228T and C250T, respectively¹³. At the top, nucleotide numbering indicates the position on chromosome 5 in the GRCh37 assembly and, at the bottom, it is related to the *TERT* translation start site. Mutations were only found at the C228T “hot spot” site in 15% of MPM. Representative sequence chromatograms of wild-type MPM and mutated MPM with cytosine-to-thymine transition are shown. The sequence and the chromatograms are represented on the minus strand of genomic DNA.

On the right, the number of MPM with mutation at the C228T “hot spot” site are given in MPM in culture and tumor samples.

Figure 3. Comparison of *TERT* promoter mutation and *TERT* gene expression in MPM.

TERT mRNA expression, measured by qRT-PCR as described in [Figure 1](#), is shown for MPM with and without mutation in the *TERT* promoter. The box plots indicate the distribution of mRNA levels in MPM in culture ([a](#)) and MPM tumor samples ([b](#)). *P*-values, determined by the Mann-Whitney test, are indicated above each dataset. Data show a significant overexpression of the *TERT* gene in MPM with mutation in the *TERT* promoter.

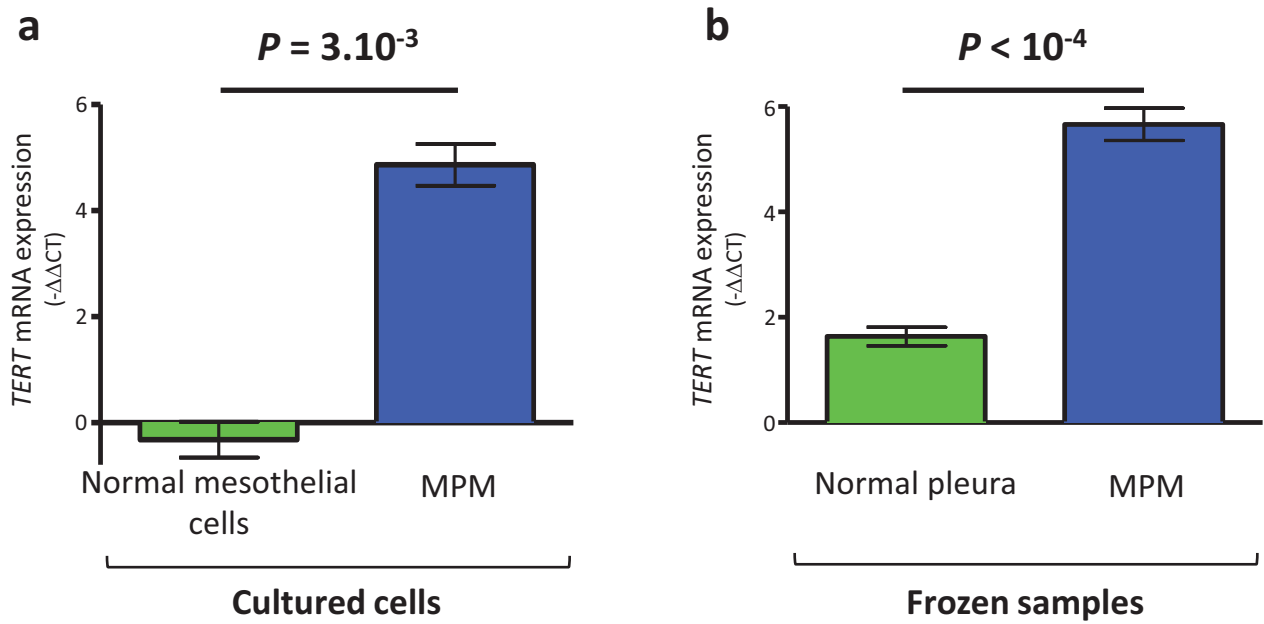


Figure 1

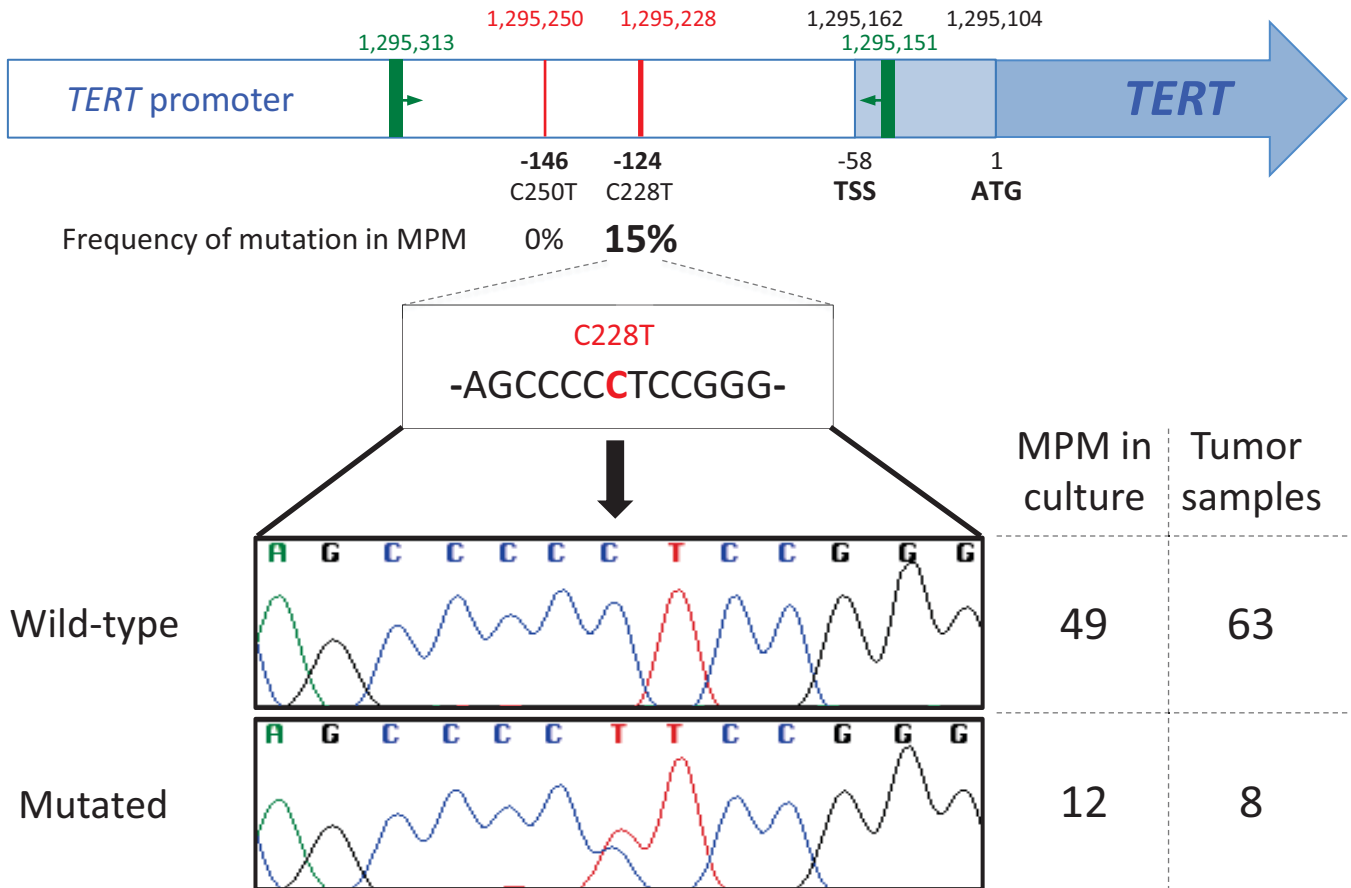


Figure 2

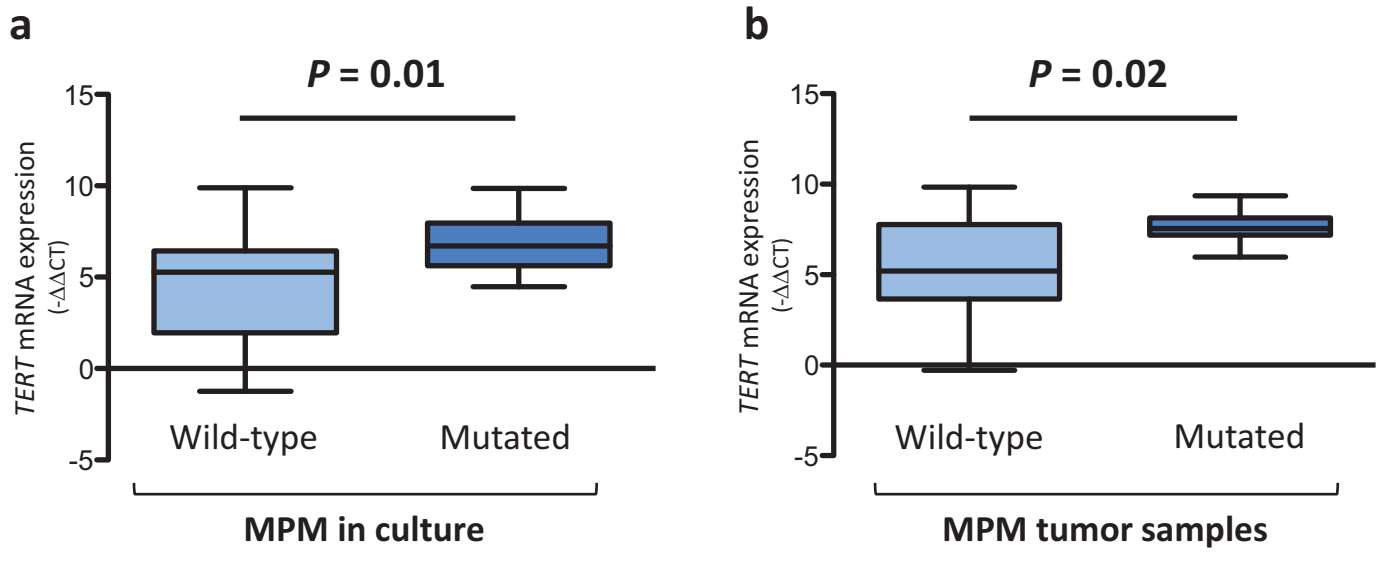


Figure 3

Table 1. Characteristics of MPM with *TERT* promoter mutation.

	<i>TERT</i> promoter		<i>P</i> -value*
	wt	mut	
Histologic subtypes n [%]	MPM in culture and tumor samples		
	N=107	N=19	
Epithelioid	85 [89]	10 [11]	8.10 ⁻³
Sarcomatoid/Desmoplastic	9 [60]	6 [40]	
Biphasic	13 [81]	3 [19]	
Genetic alterations n [%]	MPM in culture		
	N=49	N=12	
<i>CDKN2A</i>	34 [69]	12 [100]	0.03
<i>CDKN2B</i>	30 [61]	11 [92]	0.08
<i>NF2</i>	18 [37]	8 [67]	0.10
<i>BAP1</i>	27 [55]	3 [25]	0.10
<i>TP53</i>	5 [10]	1 [8]	1.00

* Fisher exact test determined using GraphPad prism software [version 5.0b]