

***KLF6* and *TP53* mutations are a rare event in prostate cancer: distinguishing between Taq polymerase artifacts and true mutations**

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Krüppel-like factor 6 (*KLF6*) has been reported to act as a tumor suppressor gene involved in the regulation of the cell cycle by activating p21 in a p53-independent manner. Many studies suggest that *KLF6* is inactivated by allelic loss and somatic mutation. However, there is a high variability in the reported frequency of mutations (from 1 to 55%). *TP53* also regulates the cell cycle through the activation of p21. In prostate cancer, the reported frequency of *TP53* mutations ranges from 3 to 42%. In all these reports, there is a considerable degree of methodological heterogeneity. Our aim was to determine the frequency of *KLF6* and *TP53* mutations in a well-defined group of prostate tumors with different stages and Gleason grades. The four exons of *KLF6* and exons 4–9 of *TP53* were studied in 103 cases, including 90 formalin-fixed, paraffin-embedded (FFPE) and 13 frozen samples. All tumors were analyzed through PCR and direct sequencing. All changes found were confirmed by a second independent PCR and sequencing reaction. For *KLF6*, mutation (E227G) was only detected in one tumor (1%) and for *TP53*, three different mutations (L130H, H214R, and Y234C) were detected in five tumors (5%). This low mutation index is in keeping with recent papers on the subject. Our study strongly supports the notion that *KLF6* and *TP53* mutations are not frequent events in prostate cancer. When using FFPE tissues, it is mandatory to perform at least two independent rounds of PCR and sequencing to confirm mutations and exclude Taq polymerase-induced artifacts.

Modern Pathology (2008) 21, 1470–1478; doi:10.1038/modpathol.2008.145; published online 19 September 2008

Keywords: *KLF6*; *TP53*; prostate cancer; mutations; artifacts

Prostate cancer is the third most diagnosed cancer in Spain,¹ and it is the second leading cause of cancer death in American men.² Many different genes have been investigated for their potential roles in the development and progression of prostate cancer.^{3,4} Two of these genes are Krüppel-like factor 6 (*KLF6*) and *TP53*.

KLF6 is a transcription factor that interacts with DNA through three zinc-fingers in its COOH-terminal domain. *KLF6* belongs to the KLF family, a family that is broadly involved in cell differentiation, development, growth-related signal transduc-

tion, cell proliferation, apoptosis, and angiogenesis.⁵ It has been suggested that *KLF6* could be involved in the regulation of the cell cycle by activating p21 in a p53-independent manner.⁶ This has been proven in several *in vitro*⁵ and *in vivo* assays.⁷ *KLF6* is believed to regulate cancer development and progression through the downregulation of the c-Jun oncoprotein⁸ and the activation of E-cadherin.⁹

The reported frequency of *KLF6* mutations in different types of human cancer varies in the different published studies.^{10–29} In prostate cancer, four studies have analyzed the frequency of *KLF6* mutations.^{6,30–32} In the first published study, Narla *et al*⁶ showed a very high frequency, 55%, whereas in the following study, Chen *et al*³⁰ obtained a lower frequency, around 15%. The results of two recent reports provided frequencies of 0 and 1%.^{31,32} It should be noted that there were considerable

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Received 30 May 2008; revised and accepted 6 August 2008; published online 19 September 2008

differences in both the selected cohorts and the methodological approaches of these studies, which could, at least in part, explain the discrepancies in their results.

On the other hand, the reported frequency of loss of heterozygosity (LOH) in prostate cancer ranges from 28 to 77%.^{6,30} *KLF6* promoter hypermethylation has been suggested as another possible inactivating mechanism,^{23,33–35} although according to some authors³⁵ epigenetic alterations are not relevant in prostate cancer. Other studies have reported downregulation of *KLF6* mRNA levels in hepatocellular carcinoma (HCC) and in lung cancer cell lines, as well as in prostate cancer cell lines. Decreased expression of *KLF6* has been associated with poor prognosis in lung adenocarcinoma and prostate cancer.^{30,36–39}

The role that *KLF6* polymorphisms play in increasing the risk of developing prostate cancer is also controversial. Narla *et al*⁴⁰ proposed that the presence of a germline single-nucleotide polymorphism (SNP) (IVS1 -27G>A) in *KLF6* could produce a splicing variant that would reduce the activation of p21. This polymorphism was associated with an increased risk for prostate cancer,⁴⁰ but subsequent reports have shown contradictory results.^{32,41–44} Moreover, the R201R polymorphism of *KLF6* has been reported to be of marginal importance in the predisposition of developing prostate cancer in a series of Finnish hereditary prostate cancer patients.⁴⁵

The tumor suppressor gene *TP53* acts in response to diverse cell stress situations by regulating target genes that induce cell-cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism.⁴⁶ Like *KLF6*, *TP53* also regulates the cell cycle through the activation of p21.⁴⁷ Mutations of *TP53* occur in half of all human tumors.⁴⁸ In prostate cancer, the reported frequency of mutation of *TP53* in the different subgroups of prostate cancer is not well defined and ranges from 3 to 42%.^{2,49} The vast majority of *TP53* mutations are missense point mutations⁵⁰ that affect the DNA-binding domain.⁵¹ Many authors have used immunohistochemistry to evaluate the *TP53* gene status, because some mutations could lead to p53 protein accumulation and immunohistochemical detection. Nevertheless, immunohistochemical overexpression does not necessarily indicate gene mutation.² The amount of p53 protein in cells is determined mainly by the rate at which it is degraded through the activity of *HMDM2*. This process is related to a feedback loop, as p53 activates the transcription of *HMDM2*, and *HMDM2* stimulates p53 degradation.⁵² It has also been reported that mutations in *TP53* and *CHEK2*, a gene activated in response to various DNA-damaging agents, could be mutually exclusive in 25% of prostate tumors.⁵³

From all these studies, it is obvious that, although *KLF6* and *TP53* are important in controlling the cell cycle, their involvement in the pathogenesis of

prostate cancer is still controversial and uncertain. Thus, the main goal of the present study has been to elucidate the real frequency of *KLF6* and *TP53* mutations in a large group of prostate cancer samples classified by stage and Gleason grade and performing more than one round of PCR and sequencing, in order to avoid artifactual mutations and other methodological problems often encountered in formalin-fixed, paraffin-embedded (FFPE) samples.

Materials and methods

Tumor Samples and Patients

A total of 103 cases of prostate cancer selected from the files of the Department of Pathology at the Hospital del Mar in Barcelona, Spain, are the subject of this report. None of the patients received chemotherapy or hormonal therapy. A total of 90 of them were FFPE samples and 13 were frozen tissues, and none of them were matched. From these 103 cases, 77 were biopsy or prostatectomy specimens, 16 were tumors found at autopsy, so-called latent tumors, 8 were bone, and 2 lymph node metastases. Together, the Gleason grades of the biopsy, surgical, and autopsy specimens were: ≤ 6 in 30 cases, 7 in 36 cases, and ≥ 8 in 27 cases. In the surgical cases, TNM classification was performed whenever possible. Pathologic stage was pT2a in 6 cases, pT2b in 11 cases, pT3a in 16 cases, and pT3b in 2 cases. Prostatectomy specimens had been completely embedded, and with the tumor foci marked in every section, a map was obtained. Tumor volume was estimated by measuring the main diameters of the foci, considering the number of cross-sections in which they were present and the standard correction factor of 0.4. Many of the surgical cases with Gleason grade 6 had tumor volume greater than 0.5 cm³, and some had extra-prostatic involvement or positive margins, features that excluded them from the standard definition of 'insignificant' prostate cancer (ie, organ-confined tumors, with tumor volume 0.5 cm³ and combined Gleason score 6 with no pattern 4). In a small subset of eight cases, found by needle biopsy and with Gleason 3 + 3, no surgical treatment was performed, and therefore, we lacked any detailed staging information (Supplementary Table 1). Two prostate cell lines (PC3 and DU145) were also included as controls, *TP53* and *KLF6* mutations have been reported in these cell lines.

KLF6 and *TP53* Mutation Analysis

Representative tumor areas containing at least 50% and usually around 90% tumor cells were selected and manually microdissected from FFPE tissue sections or frozen tissues. Standard H&E slides

served as templates. DNA was extracted using the DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) from two to three consecutive 10 µm sections. To avoid the risk of PCR contamination, genomic DNA was extracted in a laboratory where neither PCR reactions were performed nor PCR products handled. *KLF6* exons 1–4 and *TP53* exons 5–8 were amplified by PCR and directly sequenced in all cases. In the 13 cryopreserved samples, exons 4 and 9 of *TP53* were also analyzed. As exon 2 of *KLF6* is very long, it was divided into three regions (2A, 2B, and 2C). The primer sequences and the size of the products used are shown in Table 1. PCR reactions were performed in a 25 µl volume using 10–50 ng of DNA, 0.2 µmol/l for each primer, 200 µmol/l deoxynucleotide triphosphates, 3.5 mmol/l MgCl₂, 1 × PCR II buffer, and 1.5 U of Amplitaq Gold DNA Polymerase (PerkinElmer Applied Biosystems, Foster City, CA, USA). PCR conditions were as follows: 95°C (5 min) for 1 cycle, 95°C (40 s), 63°C (40 s), 72°C (40 s) for 40 cycles, and a final extension step of 72°C (5 min). PCR products were separated by electrophoresis and visualized with ethidium bromide. Samples without DNA templates were included in all assays as negative controls. PCR products were purified using the Qiagen PCR purification kit (Qiagen, Crawley, UK) according to the manufacturer's protocol.

Table 1 Primer sequences, amplified fragment size, and primers' annealing temperatures for *KLF6* and *TP53*

Exon	Primer sequence	Fragment (bp)
<i>KLF6</i>		
1	F TCCGGGGAGACTTTCGGCTC	204
	R CTGTCCGCCGGCTGCGTTTA	
2A	F AATCACGTGCCTTCTCTGGT	289
	R AAAGTTCCTCGGAGCTGTCA	
2B	F GATGTCAGCAGCGAATCCTC	251
	R TCGCCATTTCCCTTGTCAACC	
2C	F GCGGGACTTCGGGGGAAGCCA	168
	R AATGCAGTGGCGCCACCAG	
3	F AGTGAAGACATGGGCTGCTT	236
	R GCATTGTCTCAGGCACGTA	
4	F GCAAGGGATGGGAACCTAAC	102
	R CTCTCAGCCTGGAAGCCTTT	
<i>TP53</i>		
4	F CACCCATCTAACAGTCCCCCTTG	307
	R CTTGCACGGTCAGTTGCCCTGAG	
5	F TTTCAACTCTGTCTCCTTCTCT	250
	R TGGGCAACCAGCCCTGTC	
6	F ACGACAGGGCTGGTTGCCCG	200
	R CTCCCAGAGACCCAGTT	
7	F CCTCATCTTGGGCCTGTCTT	210
	R CCAGGGTTCAGCGGCAAGCA	
8	F CTGCTCTTGCTTCTCTTTT	190
	R TCTCCTCCACCGCTTCTTGT	
9	F TTATGCTCAGATTCACCTTTAT	212
	R TGAGCTGTTTACCTGCAATTG	

KLF6, Krüppel-like factor 6.

Non-autopsy cases were collected between 2000 and 2007, and autopsy cases were collected between 1993 and 2006. As most autopsy cases were relatively old, we checked the quality of the DNA by performing a previous control PCR. This multiplex PCR reaction consisted in the amplification of fragments of 100, 200, 300, 400, and 600 bp of the β-actin gene.

Mutation analysis was performed by direct sequencing of purified PCR products with the Big Dye Terminator Kit v.3.1 (PerkinElmer Applied Biosystems) using an ABI PRISM 377 instrument (PerkinElmer Applied Biosystems). Each PCR product was sequenced in both forward and reverse directions. The same sets of primers used for PCR amplification were also used for sequencing. In all cases where a change was identified in the first PCR reaction (42% of the cases for *KLF6* and 28% for *TP53*), an independent PCR amplification and sequencing was performed using the same DNA sample to verify the results. When we identified a new change in this second PCR, we performed a third, independent amplification and sequencing experiment. We considered there to be a confirmed mutation when we found the same change in two independent PCR and sequencing rounds. Taking the first, second, and third independent amplifications of *KLF6* and *TP53* together, the reproducibility of our mutational analysis was about 97%. In some cases, the mutational study could not be completed due to a failure of PCR reaction or small sample size. For this reason, the *KLF6* exon 2A could not be amplified in 10 cases, exon 2B in 5 cases, and exon 3 in 8 cases. For *TP53*, exon 5 could not be amplified in 6 cases, exon 6 in 10 cases, exon 7 in 7 cases, and exon 8 in 7 cases. In summary, amplification was not feasible for 27 exons of *KLF6* in 15 cases and for 30 exons of *TP53* in 17 cases.

Results

For *KLF6*, a total of 43 changes were found in different exons in the first PCR amplification, 42 in the DNA from FFPE samples, and 1 in the DNA from the frozen samples. In the second independent PCR reaction and sequencing, only five changes were found at exactly the same point. Four of them were confirmed in the FFPE specimens (4 of 42, 9.5%), whereas the only mutation detected in frozen tissue was also confirmed in the second PCR. The other changes (38 of 43, 88.3%) were not confirmed by the second PCR and sequencing reaction.

For *TP53*, 29 changes were detected in the first PCR amplification and sequencing reaction. Only 5 changes (17.2%) were confirmed in a second independent PCR reaction and sequencing (5 of 29). No changes in *TP53* were found in the frozen samples.

Thus, the overall frequency of artifacts was 36.9% (38/103) for *KLF6* and 23.3% (24/103) for *TP53*. All

the changes not confirmed in a second PCR and sequencing reaction were found in FFPE cases.

The analysis of the four exons of *KLF6* in the 103 cases revealed two different confirmed genetic mutations in five different tumors. The first change was a missense mutation detected only in one case and generating the transition T→C at codon 227 (Figure 1). This E227G mutation was located in exon

3, and the tumor had a Gleason grade=6. This mutation has not been reported in previous studies. The other change was detected in exon 1 on the 5'-UTR region (-4C>A) (Table 2). This change was detected in two independent PCR and sequencing reactions in four different tumors: two bone metastases, one found in a radical prostatectomy tumor with Gleason grade 8, and one from a grade 6. The

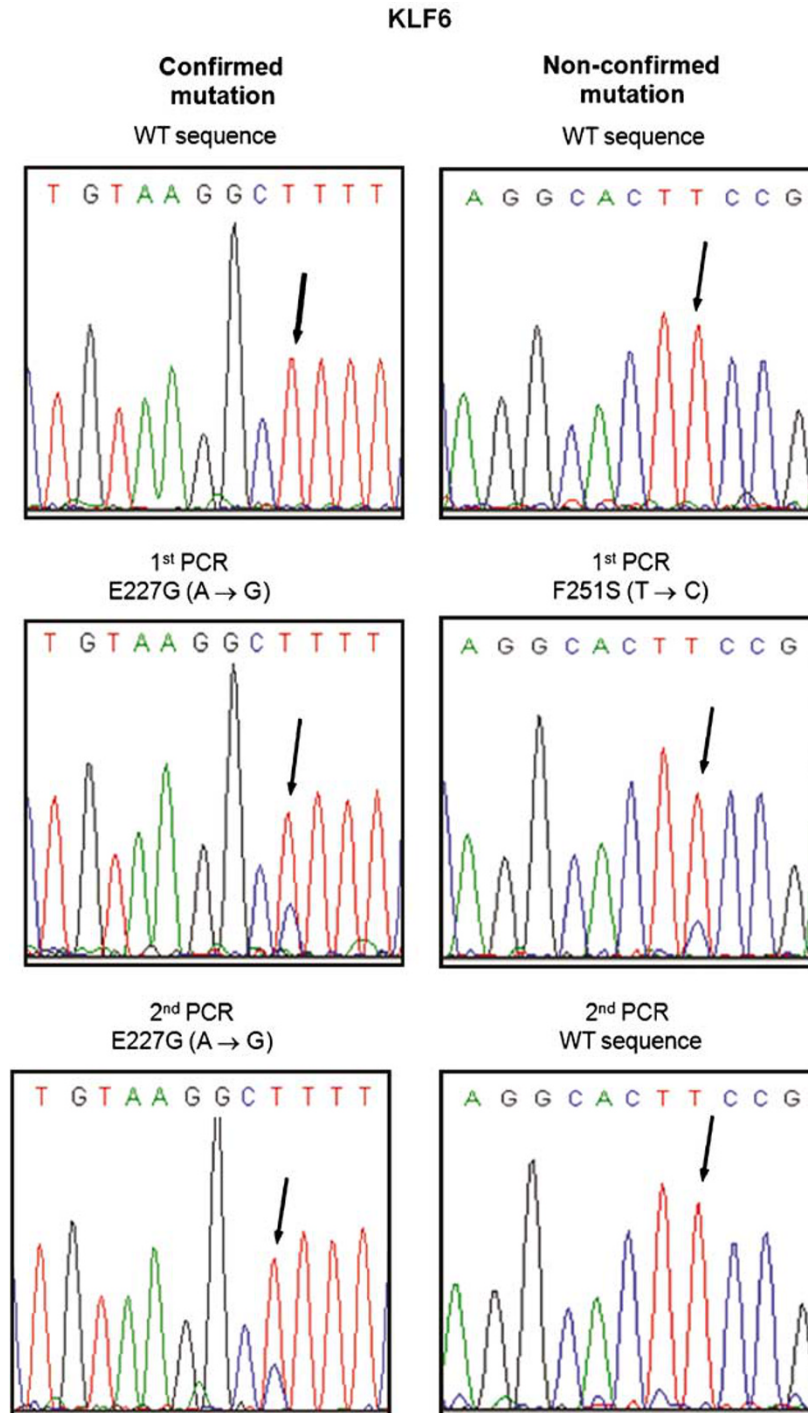


Figure 1 KLF6 mutation. The change is indicated by an arrow. There was a mutation in exon 3, transition T→C at codon 227.

Table 2 Summary of mutations found in *KLF6* and *TP53*

Case	Gene	Exon	Genetic change	Type of mutation	Gleason score	Type of sample	FFPE/Fresh tissue
1	<i>KLF6</i>	Exon 3	E227G	Missense	3+3	Clinically significant tumor	FFPE
2	<i>KLF6</i>	5'-UTR	-4C>A	—	3+3	Clinically significant tumor	Fresh tissue
3	<i>KLF6</i>	5'-UTR	-4C>A	—	4+4	Clinically significant tumor	FFPE
4	<i>KLF6</i>	5'-UTR	-4C>A	—	—	Bone metastases	FFPE
5	<i>KLF6</i>	5'-UTR	-4C>A	—	—	Bone metastases	FFPE
6	<i>TP53</i>	Exon 5	L130H	Missense	4+3	Clinically significant tumor	FFPE
7	<i>TP53</i>	Exon 5	L130H	Missense	4+4	Clinically significant tumor	FFPE
8	<i>TP53</i>	Exon 5	L130H	Missense	3+3	Autopsy	FFPE
9	<i>TP53</i>	Exon 6	H214R	Missense	4+5	Clinically significant tumor	FFPE
10	<i>TP53</i>	Exon 7	Y234C	Missense	3+4	Autopsy	FFPE
11	<i>TP53</i>	Exon 6	P223L	Missense	—	DU145	Cell line
12	<i>TP53</i>	Exon 8	V274F	Missense	—	DU145	Cell line

KLF6, Krüppel-like factor 6.

exon 1 -4C>A change was previously reported in LAPC-3 and LUCaP49 xenografts by Chen *et al.*³⁰ Bar-Shira *et al.*³² described this alteration as a polymorphism, as it was detected in a control population and no statistical difference was found between prostate tumors and control samples.

No mutations in *KLF6* were detected in prostate cell lines PC3 and DU145. This is concordant with previous reports,³¹ except for Chen *et al.*³⁰ who reported a mutation -29A>G in the 5'-UTR of the PC3 cell line.

For *TP53*, three different mutations were identified in five different tumors. Three cases, with Gleason grades 6, 7, and 8, respectively, had the transversion T→A, resulting in the mutation L130H in exon 5. The other two cases harbored the transition A→G, resulting in the mutations H214R in a case with Gleason 9 (Figure 2) and Y234C in a case with Gleason 7 in exons 6 and 7, respectively (Table 2). All the mutations occurred at codons previously reported to be mutated in prostate cancer, as well as in other cancer types (www.iarc.fr). Finally, in 8 of the 13 frozen samples, in which exons 4–9 were also analyzed, the previously reported R72P (G→C) polymorphism was detected (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1042522). Three cases were homozygous for that change and five were heterozygous.

In the prostate cancer cell line PC3, no *TP53* mutations were detected, but in the DU145 cell line, two previously reported mutations were found.^{54–57} They consisted of a transition C→T in codon 223 of exon 6, generating the missense mutation P223L and a transversion G→T in codon 274 of exon 8, resulting in the missense mutation V274F.

The analysis of *KLF6* and *TP53* revealed one and five truly mutated tumors, respectively. The remaining changes, 38 in *KLF6* and 24 in *TP53*, were artifacts. Most of these non-confirmed mutations involved the change of an adenine, suggesting that they could be artifactual mutations generated by Taq polymerase in the amplification process on FFPE samples.

Discussion

The described analysis of the four exons of *KLF6* in the 103 cases revealed only one real mutation, which generated the transition T→C at codon 227 (E227G). The extensive study of exons 5–8 of *TP53* showed three different mutations in five different tumors (L130H, H214R, and Y234C). The results obtained in the present study showed a very low frequency of mutation in *KLF6* (<1%) and also a low frequency in *TP53* (≈5%). The global rate was approximately 6%, with 6 out of 103 cases harboring mutations. Most of the mutations occurred in cases with a Gleason score of 7 or more, indicating that although *KLF6* and *TP53* are not prevalent in prostate cancer, they tend to occur in tumors with high Gleason scores.

A polymorphism -4C>A 5'-UTR was detected in *KLF6* in four (4%) different cases. Finally, a polymorphism (R72P) was identified in *TP53* in eight (8%) tumors.

In the case of *KLF6* the results are in concordance with the most recently published papers on *KLF6* mutations in prostate cancer,^{31,32} and they contrast with the much higher frequencies found in earlier studies.^{6,30} The rigorous methodological approach of our study strongly supports that many previously reported *KLF6* mutations were probably artifacts.

It is surprising that, whereas in their initial paper Narla *et al.*⁶ reported that about half of their tumors had mutations in the *KLF6* gene, Chen *et al.*³⁰ subsequently found a lower frequency with only 15% mutations. Moreover, in the third published study, Mühlbauer *et al.*³¹ found no *KLF6* mutations in any of their 32 cases. Finally, using a different approach, Bar-Shira *et al.*³² reported a frequency of mutations of less than 1% with only two mutations in 300 cases. Furthermore, all of the mutations identified in these studies were different. Only Mühlbauer *et al.*³¹ confirmed mutations by performing two independent amplification PCRs. In contrast with the other studies, they only analyzed exon 2. None of the other studies included methods to

TP53

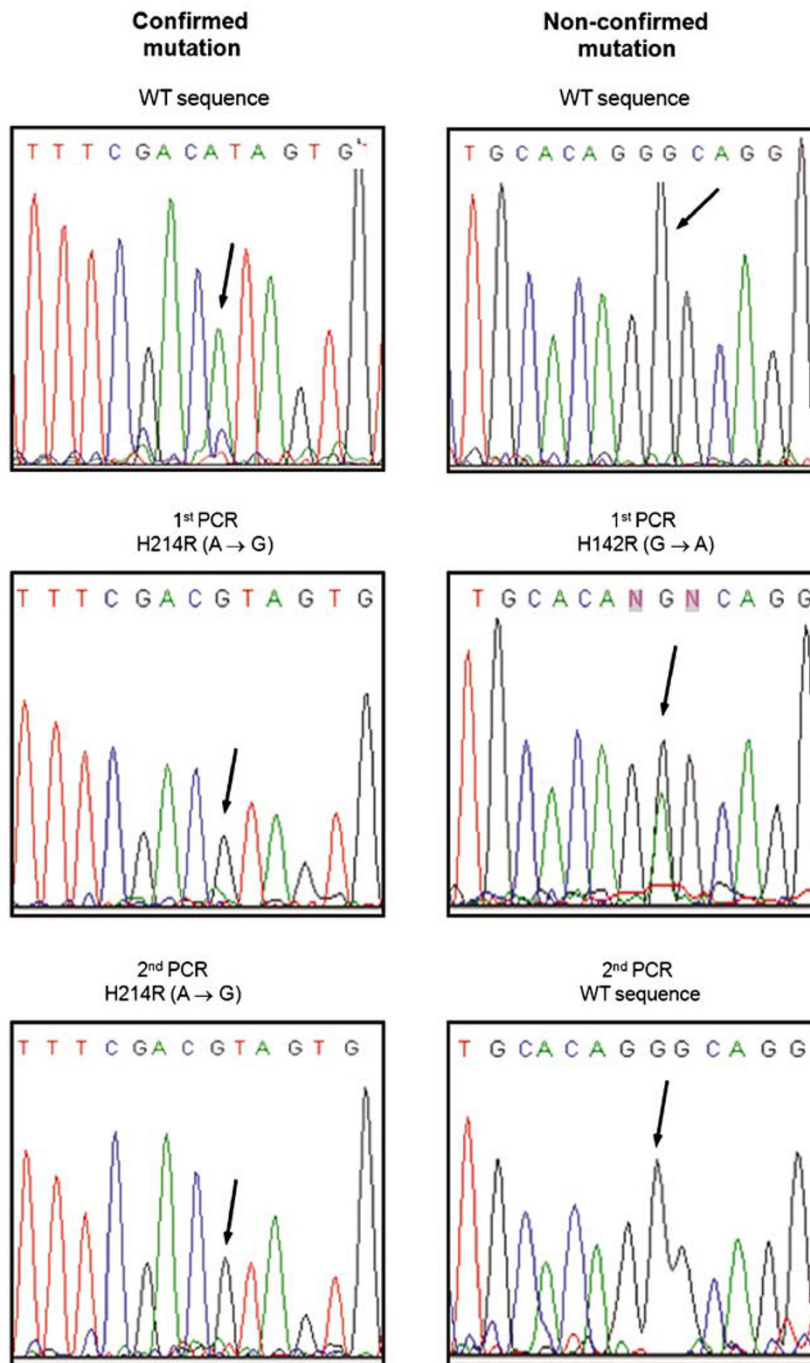


Figure 2 TP53 mutation. The change is indicated by an arrow. There was a mutation in exon 6, transition A→G at codon 214.

compensate for the possibility of artifacts being introduced by Taq polymerase. Although Chen *et al*³⁰ repeated the single strand conformation polymorphism (SSCP) analysis in the cases with band shift in DNA, sequencing was performed only once for one PCR product.

Discrepancies in the frequency of *KLF6* mutations have also been observed in HCC^{10–13} (ranging between 0 and 15%) and colorectal carcinoma^{14–18} (ranging between 0 and 44%). Similar to prostate

cancer, the first studies conducted on these other tumors indicated a high frequency of mutation, but this was not reproduced in subsequent reports. Song *et al*¹² in a recent study with PCR reactions performed in triplicate experiments, did not find any *KLF6* mutations in HCC. They related the controversial results in the previous series to genetic differences in the respective populations, PCR errors, or alternative splicing of *KLF6* due to a single polymorphism.

In the case of *TP53*, the most recent articles do not deal with the frequency of mutation in this gene. During the first years of *TP53* research in prostate cancer, many articles were published on this subject, and the reported frequency of mutation ranged from 3 to 42%.^{2,49} The IARC database (www.iarc.fr) reports a prevalence of 17%. Some authors have postulated that *TP53* mutations are rare in primary prostate cancer and that they are more common in prostate tumors with higher stage or grade, as well as metastases or androgen-independent tumors.² Other authors argue that *TP53* mutations are also a common event in early stages of prostate cancer development.⁵⁸ Moreover, some papers report a strong association between prostate cancer bone metastases and a high frequency of *TP53* mutations.^{2,59} In our study, no mutations were detected in metastatic tumors, but the number of metastatic cases was low. Meyers *et al*⁵⁹ performed PCR-SSCP and immunohistochemistry to detect somatic DNA alterations, but they only sequenced one case. The sensitivity of the PCR-SSCP technique to detect true mutations is lower than that of other currently used methods, such as direct sequencing. Many authors have used immunohistochemistry to evaluate the *TP53* gene mutation.^{2,59} It is thought that some mutations generate an abnormal protein that is not adequately metabolized and accumulates in the nucleus, allowing its immunohistochemical detection. In any event, immunohistochemical overexpression does not necessarily indicate gene mutation, as the protein accumulation may result from abnormalities in other genes involved in *TP53* regulation and metabolism.²

In their paper, Mühlbauer *et al*³¹ reported a 12% frequency of *TP53* mutations (3 out of 25). Their results are in keeping with the previous literature on the subject and are similar to the results of the present study.

A detailed methodological analysis of our results and those of the previous reports suggests that many of the discrepancies can be explained by technical issues. The fact that most molecular studies on prostate cancer use FFPE, whereas studies on other tumor types use frozen tissue sections as a source of DNA, is a crucial difference and must be examined with care. In the case of prostate cancer, the difficulties in retrieving fresh or frozen tumor tissue are obvious, as the gross appearance of prostate cancer is often very similar to that of normal, inflamed, or hyperplastic prostatic tissue. As a consequence, most molecular studies on prostate cancer are based on paraffin-retrieved material. This was the case in all four previous molecular studies on *KLF6*. The denaturation of DNA at AT-rich regions as a result of formaldehyde fixation, with the subsequent generation of free pyrimidine and purine residues, is well documented in the literature.^{60–63} As a result of this phenomenon, Taq polymerase tends to insert adenosines when no template base is present, thus producing artificial

mutations that are subsequently amplified throughout the PCR process. The number of errors introduced by Taq polymerase in fresh tissue samples is in the order of 1 in every 10⁵ base pairs, whereas in FFPE, it has been estimated at 1 in 500. The chances of finding the same artifact in a second independent PCR are extremely low. Therefore, this second PCR and sequencing reaction is mandatory on all FFPE samples in which a sequence change has been identified. Our results further support this notion, as all the artifacts in our cases were detected in FFPE tissue, whereas no artifacts were detected in frozen samples.

Different fixation protocols have been used to prevent DNA integrity problems.⁶⁴ However, none of the available fixatives ensures a complete preservation of DNA, and it is important to emphasize that the artifacts are induced not by fixation, but by Taq polymerase itself. In our retrospective study, we used one of the standard, buffered neutral formalin preparations. In order to exclude false-positive sequence mutations, we only accepted changes that were confirmed by two independent PCR amplifications. Interestingly, the initial frequency of mutation, including all detected changes, would have been around 42% for *KLF6* and 28% for *TP53*, a rate that is similar to those found in the early reports on both genes. In our study, most of these non-confirmed mutations (70%) were transitions or transversions involving the addition of an adenine. The impact of artifactual changes on DNA sequence is also exemplified in other reports. As such, in a previous study on *EGFR*⁶⁵ in lung cancer, many of the uncommon *EGFR* mutations found initially were discarded after performing multiple amplifications.

The different frequency of artifacts for *KLF6* and *TP53* (36.9% for *KLF6* and 23.3% for *TP53*) can be explained by the different number of base pairs (bp) amplified in each gene, namely 1250 bp for *KLF6* and 850 bp for *TP53*. Thus, the real proportion of artifacts introduced by Taq polymerase is very similar in both genes.

One of the suggested explanations for the discrepancies among the different authors relies on genetic differences in the populations studied, including diverse racial subgroups and variability in risk factors. But, in fact, this would be tenable only for very specific papers, as most of them have dealt with heterogeneous racial or geographical backgrounds.

It has recently been suggested that *KLF6* mutation may not be the only mechanism by which this gene could be involved in the pathogenesis of prostate cancer. DiFeo *et al*⁶⁶ pointed out that there are three different *KLF6*-inactivating mechanisms: LOH and somatic mutation, hypermethylation of the promoter, and dysregulated alternative splicing. They suggested that only the first and third mechanisms would be involved. Recent reports by Narla *et al*⁷ support the notion that the loss of one *KLF6* allele, even in the absence of somatic mutations in the

remaining one, could be enough to contribute to cell proliferation and tumor development. The exact mechanism by which this would be effective is still unknown. Further studies are needed to investigate alternative mechanisms by which *KLF6* could be involved in the pathogenesis of prostate cancer.

On the other hand, the low index of *TP53* mutation could probably be assumed by the hypothesis that other molecules implicated in the p53 pathway, such as HMDM2, p14^{ARF} or PI3K, may be altered.

In conclusion, the present study strongly supports the notion that *KLF6* as well as *TP53* mutations are not frequent events in prostate cancer, although they may occur in cases with a high Gleason score. Thus, the mutations in these two genes are not crucial in the pathogenesis of prostate cancer. Our study also emphasizes the need for carefully controlling DNA quality and for confirming sequence changes with independent amplification and sequencing rounds, in order to obtain reliable results in mutational studies.

Acknowledgements

Grants FIS/Instituto de Salud Carlos III 965190005 and 06/1411, from the Ministry of Health of the Spanish Government and Support Grant 2006 from the Spanish Association Against Cancer (Barcelona Territorial Board).

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