



## TP53 codon 72 polymorphism is associated with FGFR3 and RAS mutation in non-muscle-invasive bladder cancer

著者 (英)	Takashi KAWAHARA, Takahiro KOJIMA, Shuya KANDORI, Masahiro Kurobe, Takayuki Yoshino, Tomokazu KIMURA, Yoshiyuki Nagumo, Ryutaro Ishituka, Koji Mitsuzuka, Shintaro Narita, Takashi Kobayashi, Yoshiyuki Matsui, Osamu Ogawa, Mikio Sugimoto, Jun Miyazaki, Hiroyuki NISHIYAMA
journal or publication title	PLOS ONE
volume	14
number	8
page range	e0220173
year	2019-08
権利	(C) 2019 Kawahara et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
URL	<a href="http://hdl.handle.net/2241/00159450">http://hdl.handle.net/2241/00159450</a>

doi: 10.1371/journal.pone.0220173



## RESEARCH ARTICLE

# *TP53* codon 72 polymorphism is associated with *FGFR3* and *RAS* mutation in non-muscle-invasive bladder cancer

Takashi Kawahara<sup>1</sup>, Takahiro Kojima<sup>1\*</sup>, Shuya Kandori<sup>1</sup>, Masahiro Kurobe<sup>1‡</sup>, Takayuki Yoshino<sup>1</sup>, Tomokazu Kimura<sup>1</sup>, Yoshiyuki Nagumo<sup>1</sup>, Ryutaro Ishituka<sup>1</sup>, Koji Mitsuzuka<sup>2</sup>, Shintaro Narita<sup>3</sup>, Takashi Kobayashi<sup>4‡</sup>, Yoshiyuki Matsui<sup>4‡</sup>, Osamu Ogawa<sup>4</sup>, Mikio Sugimoto<sup>5</sup>, Jun Miyazaki<sup>6</sup>, Hiroyuki Nishiyama<sup>1</sup>

**1** Department of Urology, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan, **2** Department of Urology, Graduate School of Medicine, Tohoku University, Sendai, Japan, **3** Department of Urology, Graduate School of Medicine, Akita University, Akita, Japan, **4** Department of Urology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **5** Department of Urology, Faculty of Medicine, Kagawa University, Kagawa, Japan, **6** Department of Urology, School of Medicine, International University of Health and Welfare, Narita, Japan

☞ These authors contributed equally to this work.

‡ These authors also contributed equally to this work.

\* [tkojima@md.tsukuba.ac.jp](mailto:tkojima@md.tsukuba.ac.jp)



## OPEN ACCESS

**Citation:** Kawahara T, Kojima T, Kandori S, Kurobe M, Yoshino T, Kimura T, et al. (2019) *TP53* codon 72 polymorphism is associated with *FGFR3* and *RAS* mutation in non-muscle-invasive bladder cancer. PLoS ONE 14(8): e0220173. <https://doi.org/10.1371/journal.pone.0220173>

**Editor:** Masaru Katoh, National Cancer Center, JAPAN

**Received:** November 7, 2018

**Accepted:** July 10, 2019

**Published:** August 1, 2019

**Copyright:** © 2019 Kawahara et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** Gene expression data reported are available in the DDBJ databases under the accession number E-GEAD-306 and DRA008030 ([ftp://ftp.ddbj.nig.ac.jp/ddbj\\_database/gea/experiment/E-GEAD-000/](ftp://ftp.ddbj.nig.ac.jp/ddbj_database/gea/experiment/E-GEAD-000/), [ftp://ftp.ddbj.nig.ac.jp/ddbj\\_database/dra/fastq](ftp://ftp.ddbj.nig.ac.jp/ddbj_database/dra/fastq)).

**Funding:** This work was supported in part by a Grant-in-Aid for Challenging Exploratory Research (H.N.) (grant number 26670696), a Grant-in-Aid for Scientific Research B (H.N.) (grant number 26293349) and a Grant-in-Aid for Scientific

## Abstract

### Objective

*TP53*, a well-known tumor-suppressor gene in bladder carcinogenesis, has a functional single-nucleotide polymorphism on codon 72. The aim of this study was to elucidate the association between *TP53* codon 72 polymorphism and somatic mutations in bladder cancer.

### Material and methods

Germline *TP53* codon 72 polymorphism and somatic mutations of 50 cancer-associated genes were analyzed in 103 bladder cancer patients (59 non-muscle-invasive and 44 muscle-invasive), using Taqman genotyping assay and target sequencing, respectively. The expression of FGF-FGFR signaling pathway genes was analyzed by RNA sequencing of frozen tissue.

### Results

The allele frequency of *TP53* codon 72 in our cohort was 37, 42, and 21% for Arg/Arg, Arg/Pro, and Pro/Pro, respectively. Interestingly, the prevalence of *FGFR3* mutation was higher in patients with the Arg allele, whereas that of the *RAS* mutation was higher in patients without the Arg allele. The same association was seen in non-muscle-invasive bladder cancer (NMIBC) patients and no differences were observed in muscle-invasive bladder cancer patients. In NMIBC, *FGFR1* expression was higher in patients without the Arg allele and *FGFR3* expression was higher in patients with the Arg allele.

Research C (T.Kojima) (grant number 18K09158) from the Japan Society for the Promotion of Science (JSPS) (<https://www.jspa.go.jp/english/>). JSPS had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

## Conclusion

The germline *TP53* codon 72 polymorphism was associated with mutations of *FGFR3* or *RAS* and expression of *FGFR1* and *FGFR3* in NMIBC. These findings provide new insight into the molecular mechanisms underlying the influence of the genetic background on carcinogenesis in bladder cancer.

## Introduction

Bladder cancer is the fourth and twelfth most common malignancy in men and women, respectively [1]. It is particularly common in the elderly and male population. Cigarette smoking and some Chinese herbs are well-known risk factors [2–5]. Bladder cancer is derived from urothelium and progresses from non-muscle-invasive bladder cancer (NMIBC) to muscle-invasive bladder cancer (MIBC), before becoming metastatic. About 70% of bladder cancer patients have NMIBC, and the remaining 30% have MIBC or metastatic disease [6]. Molecular and histopathological features suggest that bladder cancer can develop along at least two distinct pathways. In one pathway, the papillary NMIBC develop via epithelial hyperplasia and recruitment of branching vasculature, and in the other pathway MIBC develops via flat dysplasia and carcinoma *in situ* (CIS) [2, 7]. Several important driver genes or tumor suppressor genes involved in carcinogenesis of bladder urothelium have been identified. For example, *TP53* mutations are key drivers for CIS or MIBC [8]. On the other hand, NMIBC is characterized by activating point mutations in *FGFR3* or *RAS* [9, 10], and, interestingly, activating *RAS* mutations are mutually exclusive with *FGFR3* mutations [10]. However, the underlying mechanism controlling the selection of specific somatic mutations in bladder cancer remains unknown.

*TP53* functions as a transcription factor, regulating the expression of several downstream genes, resulting in cell cycle arrest and apoptosis [11]. *TP53* is also known to have a functional single-nucleotide polymorphism (SNP) in codon 72 (rs1042522), which results in the substitution of proline (Pro) for arginine (Arg) in the proline-rich domain. *TP53* Arg72 is more potent in apoptosis induction, whereas *TP53* Pro72 is better in inducing cell cycle arrest and DNA damage repair [12–15]. *TP53* codon 72 polymorphism has been linked to an increased risk of breast cancer, cervical cancer, esophageal cancer, gastric cancer, lung cancer, and skin cancer [16]. However, studies relating to the association between *TP53* codon 72 polymorphism and bladder cancer susceptibility have shown inconclusive results [16]. We previously reported that this polymorphism affects the phenotypes or clinical outcomes of bladder cancer [17], but the underlying mechanism remains unknown.

Recently, several studies reported the interesting finding that germline SNPs affect specific somatic mutations. For instance, MC1R polymorphism affects *BRAF* mutant melanoma [18, 19], a *JAK2* germline polymorphism affects *JAK2* V617F mutant myeloproliferative neoplasms [20, 21], and *TACC3* polymorphism affects *FGFR3* mutant bladder cancer [22]. Some reports showed that the *TP53* Pro allele is associated with an increased frequency of *TP53* mutations in non-small cell lung cancer (NSCLC) [23, 24]. However, there is no report around the relationship between *TP53* codon 72 polymorphism and somatic mutations in bladder cancer.

We hypothesized that *TP53* codon 72 polymorphism could affect somatic mutations during bladder carcinogenesis and conducted this study to compare germline *TP53* codon 72 polymorphism and somatic mutations in bladder cancer. In our cohort, there was no relationship between *TP53* codon 72 polymorphism and *TP53* mutation. However, mutually exclusive

mutations of *FGFR3* and *RAS* in NMIBC were significantly related with the *TP53* codon 72 polymorphism. This finding provides new insight into the relationship between host germline polymorphism and selection for somatic mutation type in bladder carcinogenesis.

## Materials and methods

### Patients and tissue samples

This prospective multicenter cohort study included 144 patients with clinical diagnosis of urothelial carcinoma from seven institutions [25]. The research protocol was approved by the Ethics Committee of Tsukuba University Hospital (Approval number: H25-116). It was also reviewed and approved by the Ethics Committees of the following institutes: Tohoku University Hospital, Akita University Hospital, Kyoto University Hospital, Kagawa University Hospital, Hitachi General Hospital, and Tsukuba Medical Center Hospital. Tumor specimens, blood, and clinicopathological information were collected with written informed consent.

Primary bladder cancer tissue samples from 103 patients were stored as formalin-fixed paraffin embedded (FFPE) and frozen tissue. All tissue sections included malignant tumor cell nuclei in 10% or more cells of the whole specimen. The remaining 41 patients were excluded because their tumors originated in the upper urinary tract origin, were without urothelial histology, or fresh frozen tissue could not be obtained.

Hematoxylin and eosin staining was performed, and the slides were evaluated by pathologists at each institute. Tumors were staged according to the 2009 Union for International Cancer Control (UICC) 7th tumor-nodes-metastasis (TNM) classification system.

### Tumor DNA extraction from FFPE samples and mutation analysis

Tumor DNA from FFPE was extracted using a QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Limburg, Germany) according to the manufacturer's instructions. The DNA concentration was assessed using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Tumor DNA with more than 1.5 ng/ $\mu$ L, according to the Qubit fluorometer, was subjected to further analysis. In total, 10 ng DNA was used as template to generate an amplicon library for sequencing. Libraries were prepared using an Ion AmpliSeq Library Kit 2.0 and an Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies, Waltham, MA, USA), which amplifies 207 amplicons covering approximately 2800 COSMIC mutations in the following 50 cancer-associated genes in alphabetical order: *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *FRBB4*, *GNA11*, *GNAS*, *HNF1A*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KDR*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RB1*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53*, *VHL*. Adapter ligation, nick repair, and polymerase chain reaction (PCR) amplification were performed according to the manufacturer's instructions. Emulsion PCR and enrichment steps were carried out using an Ion OneTouch Template Kit and an Ion OneTouch system (Life Technologies), according to the manufacturer's instructions. Following enrichment, the amplicon libraries were sequenced with an Ion PGM Sequencer (Life Technologies). For data analysis, Torrent Suite 4.0.2 was used, and mutations were detected by the Variant Caller plugin 4.0–6 with somatic/high stringency configuration provided by Ion Torrent (Thermo Fisher Scientific).

### Germline DNA extraction and *TP53* codon 72 genotyping

Germline DNA was extracted from peripheral blood using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's instructions. Germline DNA samples were

genotyped using TaqMan single-nucleotide polymorphism genotyping assays for rs1042522 (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions. The results were analyzed on a 7500 real-time PCR system using the allelic discrimination assay program of Sequence Detection software version 1.3 (Applied Biosystems).

### RNA extraction and sequence analysis

Total RNA was extracted from frozen tissue using TRIzol (Thermo Fisher Scientific), prepared into messenger RNA (mRNA) libraries, and sequenced using Illumina NextSeq 500. Quality control, ambiguity and length trimming, mapping to the reference genome, normalization of gene expression, and evaluation of differential gene expression were performed using CLC Genomics Workbench version 10 (Qiagen). Default settings were used for quality control and ambiguity and length trimming. RNA-sequence reads were aligned to the reference genome of *Homo sapiens* GRCh38.p10 (GenBank accession number GCA\_000001405.25).

### Statistical analysis

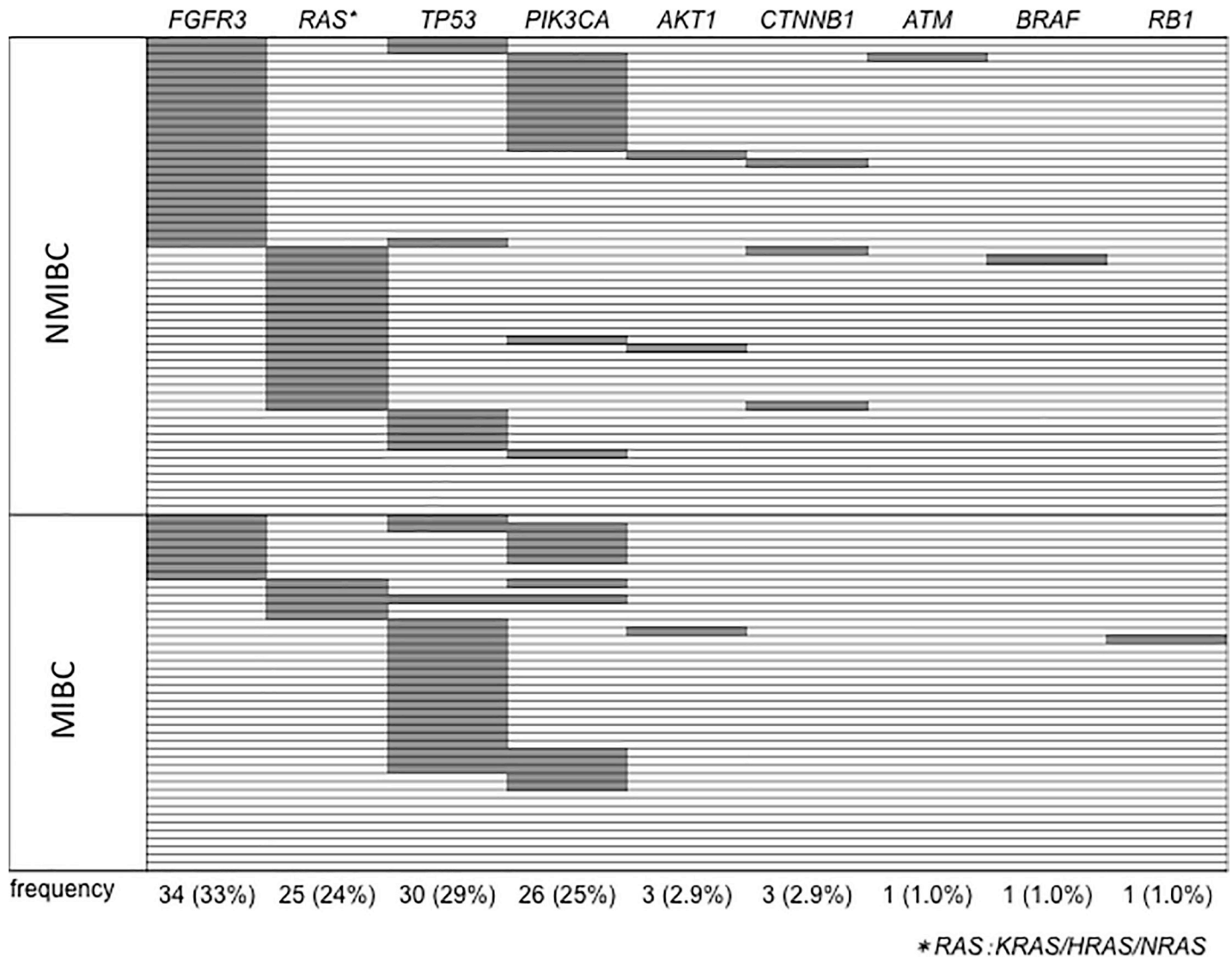
Differences were assessed using Kruskal-Wallis test and Mann-Whitney U test with Bonferroni correction. The chi-square test was used to evaluate associations between categorical variables. When the  $p$ -value was  $p < 0.05$  with chi-square test, residual analysis was performed to identify which category was significant. Gene expression was normalized using transcript per million. Wald test with Benjamini-Hochberg multiple test correction was used for evaluation of differential gene expression. Genes with false discovery rate adjusted  $p$ -values  $< 0.05$  were considered differentially expressed. Adjusted residuals were calculated with js-STAR ver 9.1.7 [26], evaluation of differential gene expression was performed using CLC Genomics Workbench version 10 (Qiagen), and the other statistical analyses were performed using STAT view ver5.0 (SAS Institute Inc. Cary, NC, USA).

## Results

### Association between germline *TP53* codon 72 polymorphism and somatic mutations in bladder cancer

Mutation analysis showed that *FGFR3*, *TP53*, *PIK3CA*, *RAS* (*HRAS*, *KRAS*, or *NRAS*), *AKT1*, *CTNNB1*, *ATM*, *BRAF*, and *RBI* mutations were present in at least one or more patients (Fig 1 and S1 Table). There were no mutations in the remaining 39 genes. The prevalence of *FGFR3* mutation was 33%, followed by *TP53* (29%), *PIK3CA* (25%), and *RAS* (24%) mutation. *FGFR3* mutation was mutually exclusive with *RAS* mutation ( $p < 0.01$ ; *RAS* mutation included *HRAS*, *KRAS*, and *NRAS*) and *TP53* mutation ( $p = 0.02$ ), but co-existent with *PIK3CA* mutation ( $p < 0.01$ ). *TP53* mutation and *RAS* mutation were also exclusive ( $p < 0.01$ ). Other mutations were not correlated with each other. The prevalence of mutations in *FGFR3* or *RAS* was higher in NMIBC than in MIBC (*FGFR3* was 51% and 25%, while *RAS* was 27% and 9.4% in NMIBC and MIBC, respectively).

Table 1 shows the frequencies of *TP53* codon 72 polymorphism. Three genotypes, Arg/Arg, Arg/Pro, and Pro/Pro, were found in 37% (38/103), 42% (43/103), and 21% (22/103) of the patients, respectively. The results fit the Hardy-Weinberg equilibrium. As shown in Table 1, clinical characteristics, including tumor grade, tumor stage, or smoking status, were not significantly different among the patients. In contrast, there was a significant difference in the prevalence of mutations in *FGFR3* and *PIK3CA* ( $p < 0.01$  and  $p = 0.04$ , respectively) among *TP53* codon 72 polymorphisms but not in *RAS* and *TP53*. Patients with Pro/Pro had significantly



**Fig 1. The frequency of somatic mutations.** Upper half shows the results of NMIBC patients, and lower half shows that of MIBC patients. We analyzed 50 cancer-related genes with Ion Ampli Seq Cancer Hotspot Panel v2 and listed the genes with mutations. Each horizontal column indicates a patient. Grey columns indicate the presence of mutation.

<https://doi.org/10.1371/journal.pone.0220173.g001>

lower *FGFR3* mutation rates and patients with Arg/Arg had higher *PIK3CA* mutation rates, as evaluated with adjusted residuals.

### Influence of Arg allele of *TP53* codon 72 polymorphism on *FGFR3* or *RAS* mutations in NMIBC

We further analyzed the allele of *TP53* polymorphism related to the prevalence of somatic mutations in bladder cancer-related genes (Table 2). When both NMIBC and MIBC patients were analyzed and divided into two groups as having Arg allele (Arg/Arg or Arg/Pro) and not having Arg allele (Pro/Pro), the prevalence of mutations in *FGFR3* was significantly higher in patients with the Arg allele (41% vs 4.5%,  $p < 0.01$ ). In contrast, the prevalence of mutations in *RAS* was higher in patients without the Arg allele (41% vs 20%,  $p = 0.04$ ). The prevalence of

Table 1. Patient characteristics and prevalence of somatic mutations with respect to the TP53 codon 72 polymorphism.

		Total, n	Arg/Arg n (%)	Arg/Pro n (%)	Pro/Pro n (%)	p-value
<b>TP53 codon 72 polymorphism</b>		<b>103</b>	<b>38 (37)</b>	<b>43 (42)</b>	<b>22 (21)</b>	
Age (year)	Median (range)	71 (39–87)	67 (41–86)	73 (39–87)	71 (60–87)	0.24**
Sex	male	85	34 (86)	33 (77)	18 (82)	0.32***
	female	18	4 (14)	10 (23)	4 (18)	
Clinical Stage	NMIBC(≤T1)	59	21 (55)	28 (65)	10 (45)	0.30***
	MIBC(≥T2)	44	17 (45)	15 (35)	12 (55)	
Grade	Low	33	12 (32)	16 (37)	5 (23)	0.49***
	High	70	26 (68)	27 (63)	17 (77)	
Smoking status	non-smoker	34	11 (30)	15 (35)	8 (36)	0.81***
	smoker	62	24 (63)	24 (56)	14 (64)	
	unknown	7	3 (8)	4 (9)	0	
Somatic mutation	FGFR3	34	16 (42)	17 (40)	1 (4.5)	< 0.01***
	RAS*	25	10 (26)	6 (14)	9 (41)	0.053***
	PIK3CA	26	15 (39)	8 (19)	3 (14)	0.04***
	TP53	30	9 (24)	15 (35)	6 (27)	0.53***

\*RAS: KRAS/HRAS/NRAS

\*\* : Kruskal-Wallis test

\*\*\*: chi-square test

<https://doi.org/10.1371/journal.pone.0220173.t001>

mutations in *PIK3CA* and *TP53* was not different between these two groups. When the patients were stratified into groups as having Pro allele (Pro/Pro or Arg/Pro) and not having Pro allele (Arg/Arg), only the prevalence of mutations in *PIK3CA* was higher in patients without the Pro allele (39% vs 17%,  $p = 0.01$ ).

Table 2. Frequency of somatic mutations divided according to the clinical stage in TP53 codon 72 polymorphism.

	Somatic Mutation	TP53 codon72 polymorphism					
		with Arg allele, n (%)	without Arg allele, n (%)	p** value	without Pro allele, n (%)	with Pro allele, n(%)	p** value
All cases	N	81	22		38	65	
	FGFR3	33 (41)	1 (4.5)	< 0.01	16 (42)	18 (28)	0.13
	RAS*	16 (20)	9 (41)	0.04	10 (26)	15 (23)	0.71
	PIK3CA	23 (28)	3 (14)	0.16	15 (39)	11 (17)	0.01
	TP53	24 (30)	6 (27)	0.83	9 (24)	21 (32)	0.35
NMIBC	n	49	10		21	38	
	FGFR3	25 (51)	1 (10)	0.02	11 (52)	15 (19)	0.34
	RAS*	13 (27)	7 (70)	< 0.01	7 (33)	13 (34)	0.95
	PIK3CA	13 (27)	1 (10)	0.26	8 (38)	6 (16)	0.054
	TP53	7 (14)	1 (10)	0.72	1 (4.8)	7 (18)	0.14
MIBC	n	32	12		17	27	
	FGFR3	8 (25)	0 (0)	0.06	5 (29)	3 (11)	0.13
	RAS*	3 (9.4)	2 (17)	0.50	3 (18)	2 (7.4)	0.30
	PIK3CA	10 (31)	2 (17)	0.33	7 (41)	5 (19)	0.10
	TP53	17 (53)	5 (42)	0.50	8 (47)	14 (52)	0.76

\*RAS: KRAS/HRAS/NRAS

\*\* : chi-square test

<https://doi.org/10.1371/journal.pone.0220173.t002>

Because mutations in *FGFR3* and *RAS* were more frequently detected in NMIBC, the prevalence of *TP53* codon 72 polymorphism and somatic mutations were separately analyzed in NMIBC and MIBC. In NMIBC, the prevalence of mutations in *FGFR3* was higher in patients with the Arg allele (51% vs 10%,  $p = 0.02$ ) and the prevalence of *RAS* mutation was significantly higher in patients without the Arg allele (70% vs 27%,  $p < 0.01$ ). In MIBC, no significant differences were identified between *TP53* codon 72 polymorphism and somatic mutations.

### ***TP53* codon 72 polymorphism could affect the expression of *FGFR1* and *FGFR3***

Since fibroblast growth factor (FGF)-FGF receptor (FGFR) signaling pathways are activated not only through FGFR gene mutation, but also with overexpression of FGF-FGFR related genes [2], the expression of all 22 subclasses of FGF (*FGF1-FGF14*, *FGF16-FGF23*) and four subclasses of FGFR (*FGFR1-FGFR4*) was determined. The association of *TP53* codon 72 polymorphism with the expression of these genes was analyzed (S2 Table). Significant differences in the expression of *FGFR1* and *FGFR3* ( $p = 0.02$  and  $p = 0.04$ , respectively) were observed, with no significant difference in the expression of other FGFs and FGFRs (S3 Table). In detail, comparing patients with Arg/Pro vs Pro/Pro, *FGFR1* expression was significantly higher in patients with Pro/Pro, and *FGFR3* expression was higher in patients with Arg/Pro ( $p^* = 0.010$  and  $p^* = 0.015$ , respectively). Comparing patients with Arg/Arg vs Pro/Pro, *FGFR1* expression tended to be higher in patients with Pro/Pro, *FGFR3* expression was not significantly different ( $p^* = 0.020$  and  $p^* = 0.38$ , respectively). Comparing patients with Arg/Arg vs Arg/Pro, neither *FGFR1* nor *FGFR3* was significantly different ( $p^* = 0.73$  and  $p^* = 0.64$ , respectively). \*:  $p$ -value was evaluated with Mann-Whitney's U test with Bonferroni correction.

We further analyzed the allele of *TP53* polymorphisms related to the expression of *FGFR1* and *FGFR3*. As shown in Table 3, when both NMIBC and MIBC patients were analyzed, *FGFR3* expression was higher in patients with the Arg allele (FDR- $p = 0.02$ ). In NMIBC patients, *FGFR3* expression was higher in patients with the Arg allele (FDR- $p = 0.02$ ). *FGFR1* expression was higher in patients without the Arg allele (FDR- $p < 0.01$ ). However, in MIBC patients, no significant differences were identified between *TP53* codon 72 polymorphism and expression of FGFRs.

**Table 3. *FGFR1/3* mRNA expression with respect to the *TP53* codon 72 polymorphism.**

Clinical stage	<i>TP53</i> codon72 polymorphism	total, n	<i>FGFR1</i> expression		<i>FGFR3</i> expression	
			mean +/- SD	FDR- $p^*$ value	mean +/- SD	FDR- $p^*$ value
All cases	with Arg allele	81	1.18 +/- 8.11	0.14	82.85 +/- 128.78	<b>0.02</b>
	without Arg allele	22	2.84 +/- 8.64		35.66 +/- 77.39	
	without Pro allele	38	1.28 +/- 8.99	0.66	63.36 +/- 97.28	1
	with Pro allele	65	1.29 +/- 7.91		54.14 +/- 135.48	
NMIBC	with Arg allele	49	0.90 +/- 2.17	<b>&lt; 0.01</b>	120.07 +/- 144.98	<b>0.02</b>
	without Arg allele	10	2.05 +/- 2.50		50.83 +/- 28.21	
	without Pro allele	21	0.90 +/- 2.32	0.79	152.85 +/- 107.17	1
	with Pro allele	38	1.01 +/- 2.25		79.26 +/- 100.57	
MIBC	with Arg allele	32	2.10 +/- 12.26	1	40.01 +/- 83.26	1
	without Arg allele	12	4.33 +/- 10.67		21.36 +/- 103.68	
	without Pro allele	17	1.90 +/- 13.11	1	34.67 +/- 79.68	1
	with Pro allele	27	3.26 +/- 11.15		25.71 +/- 94.50	

\*: Wald test with Benjamini-Hochberg multiple test correction

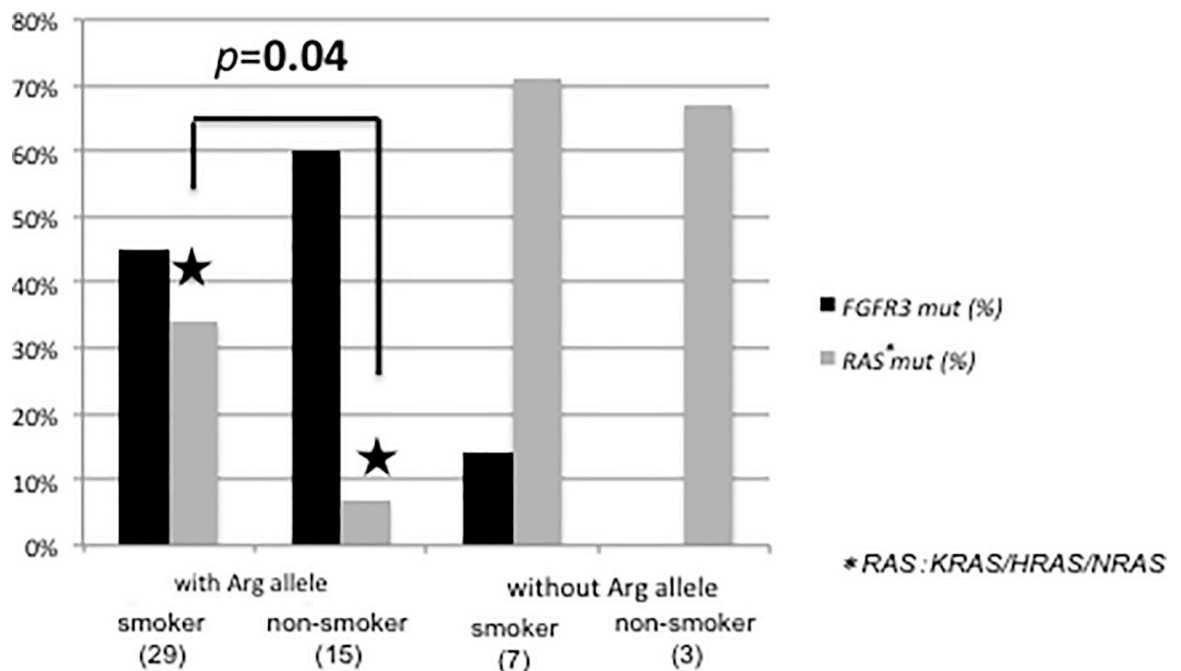


## Combination of smoking status and *TP53* codon 72 polymorphism could affect somatic mutations in NMIBC

Since smoking status has been associated with somatic mutation [27], we analyzed the frequency of *FGFR3* or *RAS* mutations in *TP53* codon 72 polymorphism and smoking status. Among NMIBC patients with the Arg allele, the prevalence of *RAS* mutation was significantly higher in smokers than in non-smokers (34% (10/29) vs 6.7% (1/15),  $p = 0.04$ ). In contrast, among NMIBC patients without the Arg allele, there was no significant difference between the prevalence of *RAS* mutations and smoking status (smokers 71% (5/7) and non-smokers 67% (2/3);  $p = 0.88$ , Fig 2). There was no significant association between smoking status and *FGFR3* or *RAS* mutations in *TP53* codon 72 polymorphism among MIBC patients.

## Discussion

In this study, we show that germline *TP53* codon 72 polymorphism could affect *FGFR3* and *RAS* mutations in NMIBC. Although there are some reports on the association between *TP53* codon 72 polymorphism and *TP53* mutation in NSCLC [23, 24], to our knowledge, there is no report on the relationship between *TP53* codon 72 polymorphism and somatic mutations, other than *TP53* mutation. In our dataset, no association between *TP53* mutation and *TP53* codon 72 polymorphism was observed (Tables 1 and 2). However, patients with the Arg allele were associated with *FGFR3* mutation and patients without the Arg allele were associated with *RAS* mutation in NMIBC (Table 2). To the best of our knowledge, this is the first report clarifying the relationship between germline *TP53* codon 72 polymorphism and somatic mutations in bladder cancer. Several studies have identified that germline polymorphisms are associated with specific somatic mutations [18–22]. Our results also imply that germline background could affect the specific somatic mutations in bladder cancer.



**Fig 2. Prevalence of mutations in *FGFR3* and *RAS* in *TP53* codon 72 polymorphism and smoking status of NMIBC patients.** Black bar and gray bar indicate the frequencies of *FGFR3* and *RAS* mutation, respectively. The number in parentheses indicates the patients' number.

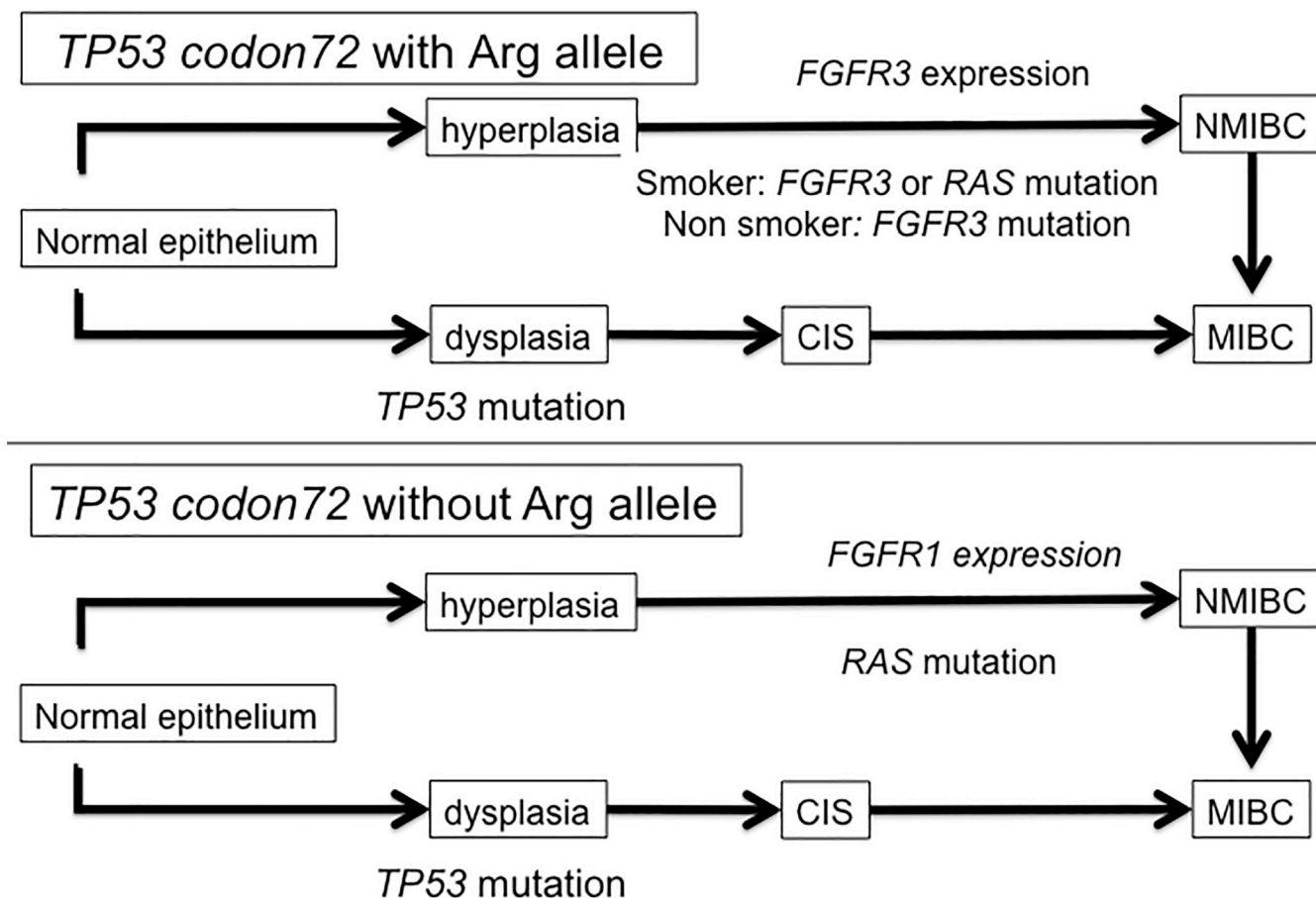
<https://doi.org/10.1371/journal.pone.0220173.g002>

The FGF-FGFR signaling pathway is composed of several subtypes of FGFs and FGFRs. Some studies have focused on activating *FGFR1* and *FGFR3* in bladder cancer [28, 29]; however, other FGFRs and FGF-ligands were not studied. Although the activation and overexpression of *FGFR3* have been reported [30], the relationship between other FGF-FGFR subtypes and their expression status have not been documented. We show that the *TP53* codon 72 polymorphism is also associated with the expression of *FGFR1* and *FGFR3*. Expression of *FGFR1* was higher in patients without the Arg allele, while expression of *FGFR3* was higher in patients with the Arg allele. Because *FGFR3* mutation activates point mutations [2], its expression was higher in patients with the Arg allele who show higher frequency of the *FGFR3* mutation. On the other hand, the reason for higher expression of *FGFR1* in patients without the Arg allele is unclear. A previous study showed that silencing *FGFR1* expression using small interfering RNA was effective in elevating *FGFR3* expression and tumor supportive activity, suggesting that *FGFR1* and *FGFR3* have an inverse relationship [31].

Several studies and the cohort presented here show that *FGFR3* and *RAS* mutations are mutually exclusive events in bladder cancer [10, 27]. The mutual exclusivity of *FGFR3* and *RAS* gene mutations is thought to reflect activation of the same pathway. The oncogenic role of activated *FGFR3* is mediated by the activation of mitogen-activated protein kinase through the *RAS* signaling pathway [32]. *FGFR3* mutations are strongly associated with low-grade and low stage bladder cancer, with lower frequency of recurrence [33]. Unlike *FGFR3* mutations, no relation of *RAS* mutational pattern with tumor grade and stage has been found [10]. *FGFR3* mutation was seen in about 70% and *RAS* mutation in about 20% of low-grade non-invasive papillary tumors [27]. However, whether *FGFR3* or *RAS* mutation is selected in NMIBC is unclear. Our results suggest that *TP53* codon 72 polymorphism contributes to the selection of somatic mutations in NMIBC.

Several environmental or habitual factors, including smoking and inflammation, have been associated with bladder carcinogenesis. The dataset presented here suggests that smoking status could affect the somatic mutations in NMIBC, based on *TP53* codon 72 polymorphism. In patients with the Arg allele, *FGFR3* mutation was higher than *RAS* mutation. When divided into smoking status, non-smokers with the Arg allele rarely had *RAS* mutation. On the other hand, patients without the Arg allele had *RAS* mutation, regardless of smoking status. Previous reports show that smoking is associated with somatic mutations. In NSCLC, *EGFR* mutations are more frequently found in non-smokers [34, 35]. Unlike *EGFR* mutations, most *KRAS* mutated NSCLC patients are former or current smokers [36–38]. In bladder cancer, a few studies have examined the association between smoking and somatic mutations. The cancer genome atlas (TCGA) data shows no statistically significant association between smoking status and somatic mutations [39]. Pandith et al. reported that *FGFR3* and *RAS* mutations were higher in smokers, but no significant association was found [27]. TCGA analyzed only MIBC patients [39], whereas Pandith et al. analyzed both MIBC and NMIBC patients [27]. These discrepancies were probably due to analysis of different clinical background factors in patients. Nonetheless, these results suggest that different types of bladder cancer have specific mutations depending on the *TP53* codon 72 polymorphism and smoking status.

Based on the previous bladder carcinogenesis model [7] and our results, we propose a new carcinogenesis model in NMIBC (Fig 3). *FGFR3* and *RAS* mutation could be affected by the *TP53* codon 72 polymorphism and smoking status in NMIBC. Non-smokers with the Arg allele show *FGFR3* mutation and smokers with the Arg allele show either *FGFR3* or *RAS* mutation. Patients without the Arg allele show *RAS* mutation regardless of smoking status. Moreover, germline *TP53* polymorphisms could affect the expression of *FGFR1* and *FGFR3* in NMIBC. *FGFR3* expression was higher in patients with the Arg allele, and *FGFR1* expression was higher in patients without the Arg allele. Taken together these results suggest that *TP53*



**Fig 3. Proposed mechanism of two-pathway model in bladder carcinogenesis considering *TP53* polymorphism and smoking status.**

<https://doi.org/10.1371/journal.pone.0220173.g003>

codon 72 polymorphism and smoking status could affect somatic mutations and FGF-FGFR signaling in bladder carcinogenesis.

Several reports have shown that *TP53* codon 72 polymorphism is a response associated with chemotherapy or radiotherapy [40, 41]. The mechanisms underlying the influence of the *TP53* codon 72 genotype on anticancer treatment response are still unknown. Although our data show the relationship between *TP53* codon 72 polymorphism and mutations in FGF-FGFR gene expression, our results cannot explain the mechanism or the difference in treatment response among *TP53* codon 72 polymorphism.

Although our study revealed associations between *TP53* codon 72 polymorphisms and somatic mutations in bladder cancer, it was limited by the relatively small sample size. Additionally, the cohort consisted of Japanese bladder cancer patients only; there is still a lack of clarity in the underlying mechanism, and we could not identify a treatment strategy around the *TP53* codon 72 polymorphism. Therefore, studies involving larger cohorts and other ethnicities are needed to confirm our results. Nonetheless, our results contribute to the elucidation of the mechanism of bladder carcinogenesis. Further clarification regarding the relation between bladder carcinogenesis and genetic background will aid the development of bladder cancer therapy.

In conclusion, *TP53* codon 72 polymorphism is associated with *FGFR3* or *RAS* mutation in NMIBC, suggesting that host germline could affect the selection for somatic mutation type in bladder carcinogenesis.

## Supporting information

### S1 Table. Patients' metadata and mutation status.

(XLSX)

### S2 Table. FGF-FGFR expression values.

(XLSX)

### S3 Table. Comparison between expression of FGF-FGFR signaling pathway genes and *TP53* codon 72 polymorphism.

(XLSX)

## Acknowledgments

We are grateful to Dr. Naoto Keino for his valuable contributions to the collection of material and clinical information. We thank Noriko Kunita and Jun Itadani for their excellent technical assistance.

## Author Contributions

**Conceptualization:** Takahiro Kojima, Masahiro Kurobe, Koji Mitsuzuka, Shintaro Narita, Takashi Kobayashi, Osamu Ogawa, Mikio Sugimoto, Hiroyuki Nishiyama.

**Data curation:** Takashi Kawahara, Takahiro Kojima, Masahiro Kurobe, Koji Mitsuzuka, Shintaro Narita, Takashi Kobayashi, Yoshiyuki Matsui, Mikio Sugimoto.

**Formal analysis:** Takashi Kawahara, Takahiro Kojima, Takashi Kobayashi.

**Funding acquisition:** Takahiro Kojima, Hiroyuki Nishiyama.

**Investigation:** Takahiro Kojima, Osamu Ogawa, Hiroyuki Nishiyama.

**Methodology:** Takahiro Kojima, Osamu Ogawa, Hiroyuki Nishiyama.

**Project administration:** Takahiro Kojima, Osamu Ogawa, Hiroyuki Nishiyama.

**Resources:** Takahiro Kojima, Masahiro Kurobe, Koji Mitsuzuka, Shintaro Narita, Takashi Kobayashi, Yoshiyuki Matsui, Mikio Sugimoto, Jun Miyazaki, Hiroyuki Nishiyama.

**Supervision:** Takahiro Kojima, Osamu Ogawa, Hiroyuki Nishiyama.

**Writing – original draft:** Takashi Kawahara, Takahiro Kojima, Hiroyuki Nishiyama.

**Writing – review & editing:** Takashi Kawahara, Takahiro Kojima, Shuya Kandori, Masahiro Kurobe, Takayuki Yoshino, Tomokazu Kimura, Yoshiyuki Nagumo, Ryutaro Ishituka, Koji Mitsuzuka, Shintaro Narita, Takashi Kobayashi, Yoshiyuki Matsui, Mikio Sugimoto, Jun Miyazaki, Hiroyuki Nishiyama.

## References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin.* 2018; 68:7–30. <https://doi.org/10.3322/caac.21442> PMID: 29313949
2. Knowles MA, Hurst CD. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. *Nat Rev Cancer.* 2015; 15:25–41. <https://doi.org/10.1038/nrc3817> PMID: 25533674
3. Negri E, La Vecchia C. Epidemiology and prevention of bladder cancer. *Eur J Cancer Prev.* 2001; 10:7–14. PMID: 11263594
4. Pelucchi C, Bosetti C, Negri E, Malvezzi M, La Vecchia C. Mechanisms of disease: The epidemiology of bladder cancer. *Nat Clin Pract Urol.* 2006; 3:327–340. <https://doi.org/10.1038/ncpuro0510> PMID: 16763645

5. Chang DW, Gu J, Wu X. Germline prognostic markers for urinary bladder cancer: obstacles and opportunities. *Urol Oncol*. 2012; 30:524–532. <https://doi.org/10.1016/j.urolonc.2012.04.003> PMID: 22742565
6. Burger M, Catto JW, Dalbagni G, Grossman HB, Herr H, Karakiewicz P, et al. Epidemiology and risk factors of urothelial bladder cancer. *Eur Urol*. 2013; 63:234–241. <https://doi.org/10.1016/j.eururo.2012.07.033> PMID: 22877502
7. Bakkar AA, Wallerand H, Radvanyi F, Lahaye JB, Pissard S, Lecerf L, et al. *FGFR3* and *TP53* gene mutations define two distinct pathways in urothelial cell carcinoma of the bladder. *Cancer Res*. 2003; 63:8108–8112. PMID: 14678961
8. Fujimoto K, Yamada Y, Okajima E, Kakizoe T, Sasaki H, Sugimura T, et al. Frequent association of p53 gene mutation in invasive bladder cancer. *Cancer Res*. 1992; 52:1393–1398. PMID: 1540947
9. Billerey C, Chopin D, Aubriot-Lorton MH, Ricol D, Gil Diez de Medina S, Van Rhijn B, et al. Frequent *FGFR3* mutations in papillary non-invasive bladder (pTa) tumors. *Am J Pathol*. 2001; 158:1955–1959. [https://doi.org/10.1016/S0002-9440\(10\)64665-2](https://doi.org/10.1016/S0002-9440(10)64665-2) PMID: 11395371
10. Jebar AH, Hurst CD, Tomlinson DC, Johnston C, Taylor CF, Knowles MA. *FGFR3* and *Ras* gene mutations are mutually exclusive genetic events in urothelial cell carcinoma. *Oncogene*. 2005; 24:5218–5225. <https://doi.org/10.1038/sj.onc.1208705> PMID: 15897885
11. Olumi AF. A critical analysis of the use of p53 as a marker for management of bladder cancer. *Urol Clin North Am*. 2000; 27:75–82. PMID: 10696247
12. Dumont P, Leu JI, Della Pietra AC 3rd, George DL, Murphy M. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. *Nat Genet*. 2003; 33:357–365. <https://doi.org/10.1038/ng1093> PMID: 12567188
13. Pim D, Banks L. p53 polymorphic variants at codon 72 exert different effects on cell cycle progression. *Int J Cancer*. 2004; 108:196–199. <https://doi.org/10.1002/ijc.11548> PMID: 14639602
14. Siddique M, Sabapathy K. Trp53-dependent DNA-repair is affected by the codon 72 polymorphism. *Oncogene*. 2006; 25:3489–3500. <https://doi.org/10.1038/sj.onc.1209405> PMID: 16462765
15. Thomas M, Kalita A, Labrecque S, Pim D, Banks L, Matlashewski G. Two polymorphic variants of wild-type p53 differ biochemically and biologically. *Mol Cell Biol*. 1999; 19:1092–1100. <https://doi.org/10.1128/mcb.19.2.1092> PMID: 9891044
16. Yang Z1, Nie S, Zhu H, Wu X, Jia S, Luo Y, et al. Association of p53 Arg72Pro polymorphism with bladder cancer: a meta-analysis. *Gene*. 2013; 512:408–413. <https://doi.org/10.1016/j.gene.2012.09.085> PMID: 23073555
17. Horikawa Y, Nadaoka J, Saito M, Kumazawa T, Inoue T, Yuasa T, et al. Clinical implications of the MDM2 SNP309 and p53 Arg72Pro polymorphisms in transitional cell carcinoma of the bladder. *Oncol Rep*. 2008; 20:49–55. PMID: 18575717
18. Landi MT, Bauer J, Pfeiffer RM, Elder DE, Hulley B, Minghetti P, et al. MC1R germline variants confer risk for BRAF-mutant melanoma. *Science*. 2006; 313:521–522. <https://doi.org/10.1126/science.1127515> PMID: 16809487
19. Fargnoli MC, Pike K, Pfeiffer RM, Tsang S, Rozenblum E, Munroe DJ, et al. MC1R variants increase risk of melanomas harboring BRAF mutations. *J Invest Dermatol*. 2008; 128:2485–2490. <https://doi.org/10.1038/jid.2008.67> PMID: 18368129
20. Kilpivaara O, Mukherjee S, Schram AM, Wadleigh M, Mullally A, Ebert BL, et al. A germline JAK2 SNP is associated with predisposition to the development of JAK2(V617F)-positive myeloproliferative neoplasms. *Nat Genet*. 2009; 41:455–459. <https://doi.org/10.1038/ng.342> PMID: 19287384
21. Trifa AP, Bănescu C, Tevet M, Bojan A, Dima D, Urian L, et al. TERT rs2736100 A>C SNP and JAK2 46/1 haplotype significantly contribute to the occurrence of JAK2 V617F and CALR mutated myeloproliferative neoplasms—a multicentric study on 529 patients. *Br J Haematol*. 2016; 174:218–226. <https://doi.org/10.1111/bjh.14041> PMID: 27061303
22. Kiemeny LA, Sulem P, Besenbacher S, Vermeulen SH, Sigurdsson A, Thorleifsson G, et al. A sequence variant at 4p16.3 confers susceptibility to urinary bladder cancer. *Nat Genet*. 2010; 42:415–419. <https://doi.org/10.1038/ng.558> PMID: 20348956
23. Hu Y, McDermott MP, Ahrendt SA. The p53 codon 72 proline allele is associated with p53 gene mutations in non-small cell lung cancer. *Clin Cancer Res*. 2005; 11:2502–2509. <https://doi.org/10.1158/1078-0432.CCR-04-1913> PMID: 15814626
24. Szymanowska A, Jassem E, Dziadziuszko R, Borg A, Limon J, Kobińska-Gulida G, et al. Increased risk of non-small cell lung cancer and frequency of somatic *TP53* gene mutations in Pro72 carriers of *TP53* Arg72Pro polymorphism. *Lung Cancer*. 2006; 52:9–14. <https://doi.org/10.1016/j.lungcan.2005.12.007> PMID: 16499995

25. Kurobe M, Kojima T, Nishimura K, Kandori S, Kawahara T, Yoshino T, et al. Development of RNA-FISH Assay for Detection of Oncogenic FGFR3-TACC3 Fusion Genes in FFPE Samples. *PLoS One*. 2016; 11:e0165109. <https://doi.org/10.1371/journal.pone.0165109> PMID: 27930669
26. Tanaka S and Nakano H. js-STAR version 9. 24 July 2018. KISNET. [cited 25 January 2019]. Available from: [http://www.kisnet.or.jp/nappa/software/star/freq/chisq\\_ixj.htm](http://www.kisnet.or.jp/nappa/software/star/freq/chisq_ixj.htm).
27. Pandith AA, Hussain A, Khan MS, Shah ZA, Wani MS, Siddiqi MA. Oncogenic activation of fibroblast growth factor receptor-3 and RAS genes as non-overlapping mutual exclusive events in urinary bladder cancer. *Asian Pac J Cancer Prev*. 2016; 17:2787–2793. PMID: 27356691
28. Tomlinson DC, Lamont FR, Shnyder SD, Knowles MA. Fibroblast growth factor receptor 1 promotes proliferation and survival via activation of the mitogen-activated protein kinase pathway in bladder cancer. *Cancer Res*. 2009; 69:4613–4620. <https://doi.org/10.1158/0008-5472.CAN-08-2816> PMID: 19458078
29. Cheng T, Roth B, Choi W, Black PC, Dinney C, McConkey DJ. Fibroblast growth factor receptors-1 and -3 play distinct roles in the regulation of bladder cancer growth and metastasis: implications for therapeutic targeting. *PLoS One*. 2013; 8:e57284. <https://doi.org/10.1371/journal.pone.0057284> PMID: 23468956
30. Tomlinson DC, Baldo O, Harnden P, Knowles MA. FGFR3 protein expression and its relationship to mutation status and prognostic variables in bladder cancer. *J Pathol*. 2007; 213:91–98. <https://doi.org/10.1002/path.2207> PMID: 17668422
31. Jang JH. Reciprocal relationship in gene expression between FGFR1 and FGFR3: implication for tumorigenesis. *Oncogene*. 2005; 24:945–948. <https://doi.org/10.1038/sj.onc.1208254> PMID: 15558020
32. Juanpere N, Agell L, Lorenzo M, de Muga S, López-Vilaró L, Murillo R, et al. Mutations in FGFR3 and PIK3CA, singly or combined with RAS and AKT1, are associated with AKT but not with MAPK pathway activation in urothelial bladder cancer. *Hum Pathol*. 2012; 43:1573–1582. <https://doi.org/10.1016/j.humpath.2011.10.026> PMID: 22417847
33. Billerey C, Chopin D, Aubriot-Lorton MH, Ricol D, Gil Diez de Medina S, Van Rhijn B, et al. Frequent FGFR3 mutations in papillary non-invasive bladder (pTa) tumors. *Am J Pathol*. 2001; 158:1955–1959. [https://doi.org/10.1016/S0002-9440\(10\)64665-2](https://doi.org/10.1016/S0002-9440(10)64665-2) PMID: 11395371
34. Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst*. 2005; 97:339–346. <https://doi.org/10.1093/jnci/dji055> PMID: 15741570
35. Gazdar AF. Activating and resistance mutations of EGFR in non-small-cell lung cancer: role in clinical response to EGFR tyrosine kinase inhibitors. *Oncogene*. 2009; 28(Suppl 1):S24–31.
36. Dogan S, Shen R, Ang DC, Johnson ML, D'Angelo SP, Paik PK, et al. Molecular epidemiology of EGFR and KRAS mutations in 3,026 lung adenocarcinomas: higher susceptibility of women to smoking-related KRAS-mutant cancers. *Clin Cancer Res*. 2012; 18:6169–6177. <https://doi.org/10.1158/1078-0432.CCR-11-3265> PMID: 23014527
37. Buttitta F, Barassi F, Fresu G, Felicioni L, Chella A, Paolizzi D, et al. Mutational analysis of the HER2 gene in lung tumors from Caucasian patients: mutations are mainly present in adenocarcinomas with bronchioloalveolar features. *Int J Cancer*. 2006; 119:2586–2591. <https://doi.org/10.1002/ijc.22143> PMID: 16988931
38. Suzuki M, Shigematsu H, Iizasa T, Hiroshima K, Nakatani Y, Minna JD, et al. Exclusive mutation in epidermal growth factor receptor gene, HER-2, and KRAS, and synchronous methylation of nonsmall cell lung cancer. *Cancer*. 2006; 106:2200–2207. <https://doi.org/10.1002/cncr.21853> PMID: 16598760
39. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature*. 2014; 507:315–322. <https://doi.org/10.1038/nature12965> PMID: 24476821
40. Vivenza D, Monteverde M, Lattanzio L, Tonissi F, Astesana V, Denaro N, et al. Correlation of TP53 and MDM2 genotypes and clinical outcome in platinum-treated head and neck cancer patients with more than 10 years' follow-up. *Int J Biol Markers*. 2016; 31:e183–192. <https://doi.org/10.5301/ijbm.5000192> PMID: 26916894
41. Liu Y, Wang X, Ding N, Mi L, Ping L, Jin X, et al. TP53 Arg72 as a favorable prognostic factor for Chinese diffuse large B-cell lymphoma patients treated with CHOP. *BMC Cancer*. 2017; 17:743. <https://doi.org/10.1186/s12885-017-3760-0> PMID: 29126407