Development of a high-resolution melting method for the screening of *TNFAIP3* gene mutations

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Abstract. Tumor necrosis factor, α-induced protein 3 (*TNFAIP3)* which encodes a ubiquitin-modifying enzyme (A20), acts as a negative regulator of the NF-κB pathway, and in lymphoma and autoimmune diseases it is frequently inactivated by mutations and/or deletions. We investigated the prevalence of the inactivation of *TNFAIP3* in oral squamous cell carcinoma (OSCC). DNA was extracted from 81 cases of OSCC and 50 peripheral blood samples from normal controls. A high-resolution melting (HRM) analysis was used to characterize *TNFAIP3* mutations, and the results were confirmed by direct DNA sequencing. Three mutations and three single-nucleotide polymorphisms (SNPs) were found to be associated with OSCC; the *TNFAIP3* mutation occurred in 3.7% (3/81) of the OSCC cases examined. All mutations were in exon 7 [c.1081G>A (p.E361K), c.1398C>G (p.S466R) (rs200878487) and c.1760C>T (p.P587L) (rs150056192)], and p.E361K was identified as a novel mutation. We further used SIFT and PolyPhen-2 software to assess potentially functional mutations. Two SNPs, c.296-20_296-18delCTC (rs71670547) and c.380T>G (p.F127C) (rs2230926), were located in exon 3, and c.2140C>T (p.P714S) was located in exon 9. A novel SNP, p.P714S differed from the one reported previously (p.P714A) (rs369155845) at that site. We also identified five SNPs in 50 normal Taiwanese individuals, and two of them [c.296-15C>T (rs377482653) and c.305A>G (p.N102S) (rs146534657)] were not found in our OSCC tissue. HRM facilitated the screening of genetic changes. In addition, our results indicate that the prevalence of the *TNFAIP3* mutation is low in OSCC.

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most commonly diagnosed cancers worldwide, ranking sixth among all human cancers (1). There are 650,000 new OSCC cases diagnosed and 350,000 deaths due to the disease reported each year (2). OSCC has been one of the 10 leading causes of death from cancer in Taiwan since 1991. According to the Cancer Registry Annual Report in Taiwan, oral cancer is the sixth leading cause of cancer-related death in the entire population and fourth in the male population. The development of oral cancer is highly associated with betal-quid chewing, cigarette smoking and alcohol consumption in Taiwan (3). Although technologies for diagnosis and therapy (surgery, radiation and chemotherapy) have advanced remarkably, the long-term survival rate of OSCC patients has not improved significantly for decades (4,5).

The transcription factor NF-κB plays a key role in several cellular functions, including inflammation, innate and adaptive immune responses, cell proliferation, survival, angiogenesis and apoptosis. The constitutive activation of NF-κB is common in many types of human tumors. Dysregulation of NF-κB promotes tumor angiogenesis and metastasis as well as resistance to chemotherapeutic agents and radiation (6). Activation of the NF-κB pathway is tightly controlled by several feedback mechanisms and is regulated by ubiquitination (7).

Tumor necrosis factor, α-induced protein 3 (TNFAIP3), which encodes a ubiquitin-modifying enzyme (A20), is one of the major inhibitors of the NF-κB signaling pathway (8). It is induced by tumor necrosis factor (TNF) in human endothelial cells (9). A20 has dual functionality; it is not only able to add ubiquitin moieties to its target protein but can also cleave K63-linked polyubiquitin chains, preventing the interaction of receptor interacting serine/threonine protein kinase 1 (RIP1) and NF-κB essential modulator (NEMO) through its deubiquitination activity mediated by its ovarian tumor (OTU) domain. A20 also adds K48-linked polyubiquitin chains to RIP1, targeting it for proteasomal degradation (10).

The *TNFAIP3* gene contains eight coding exons (2-9), along with exon 1 which is non-coding, and is located on chromosome 6q23. It has been shown to be inactivated by deletions, point mutations, and/or promoter methylation in several types of lymphomas, such as B-cell lymphomas, classical Hodgkin's lymphoma, chronic lymphocytic leukemia and mucosa-associated lymphoid tissue lymphoma, which results in loss of the A20 protein (11-14). All of these lymphomas are characterized by the dysregulation of the NF-κB signaling pathway. These findings establish *TNFAIP3* as an important tumor-suppressor gene. Human genome-wide association studies (GWAS) have linked germline single nucleotide polymorphisms of the *TNFAIP3* gene with susceptibility to human inflammatory and autoimmune pathologies (15,16).

The high-resolution melting (HRM) analysis is one of the most effective mutation scanning methodologies. It is a closed-tube method, such that PCR amplification and subsequent analysis are performed sequentially in the same tube, which makes it more convenient than other scanning methodologies. Moreover, there is no need for processing or separation of PCR products (17). This study aimed to assess the utility of HRM analysis using real-time polymerase chain reaction (PCR) for screening *TNFAIP3* mutations.

Materials and methods

Patients and DNA extraction. A total of 81 patients who were recently diagnosed with OSCC were selected for the present study. Tissue specimens were stored immediately after resection in liquid nitrogen before DNA extraction. We included the peripheral blood samples of 50 unaffected individuals from the general population as controls. DNA was extracted as described previously by Yeh *et al* (18). The genomic DNA concentration was assessed using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). DNA was stored at -80˚C until use. All tumors were classified according to the TNM classification system (19). The present study was approved by the Institutional Review Board of the China Medical University Hospital (CMUH102-REC1-015).

Design of primers for HRM assay. We used a set of primers for HRM, specific for *TNFAIP3* exons 2-9 that met the requirements of the LightCycler® 480 System Gene Scanning Assay. The 13 primer pairs for HRM analysis were selected using the Primer3 software (Table I). For exons 2, 7 and 9, more than one pair of primers were used to amplify the exon in two overlapping segments. All primers synthesized were of standard molecular biology quality (Protech Technology Enterprise Co., Ltd., Taiwan).

Table I. Primers uses for HRM analysis of *TNFAIP3* gene mutations.

Detection for	Sequence	Length of PCR amplicon (bp)
Exon 2		
P2-1	F: 5'GTCAGGCTAATAGAATGGCTTTTT 3' R: 5'ATGATCTCCCGAAACTGAGGAC 3'	250
P2-2	F: 5'TTAAAACCATGCACCGATACACA 3' R: 5'CTATCACCCAGGCAAAAGAAACA 3'	218
Exon 3	F: 5'TGGGTCTTACATGCAGATAACTTG 3' R: 5'CACCATGGAGCTCTGTTAGTAGAT 3'	293
Exon 4	F: 5'AGGGAGTACAGGATACATTCAAGC 3' R: 5'AAGGCTGAAAGCATTTAAGTACAGA 3'	245
Exon 5	F: 5'ATGGAATTTGATGAAAGTCACCTA 3' R: 5'AAGGAAAACCCTGATGTTTCAGT 3'	289
Exon 6	F: 5'TGAGATCTACTTACCTATGGCCTTG 3' R: 5'GACACAGGAGAGAGCTGAACATAA 3'	283
Exon 7		
P7-1	F: 5'TGTAAAATCTTGTGTGTGATTTTGTG 3' R: 5'CTCTGAGCACTCATGGCATAAAG 3'	302
P7-2	F: 5'CCTTCTTCATGTCTGTGAACACC 3' R: 5'CAACGTTCACAAAATCCGTTGT 3'	316
P7-3	F: 5'AGTGAGACCACTGCCATGAAG 3' R: 5'TTCCAGCTCTGTGGCAAGAAT 3'	340
P7-4	F: 5'CACCAGCGTTCCAAGTCAGAT 3' R: 5'TTCTTAAAGGTCAGGAACAAAACC 3'	301
Exon 8	F: 5'TCTACTGTCAGCATCTCTGTATCG 3' R: 5'AGCAAAAAGCATCGAACACAC 3'	307
Exon 9		
P9-1	F: 5'AGATTTCATTGTGCTCTCCCTAAG 3' R: 5'CTGGTTGGGATGCTGACACT 3'	215
P9-2	F: 5'GCCTCCTGCAAGAACATCCT 3' R: 5'ATAGCACCATGATGACTGACAGC 3'	285

F, forward; R, reverse.

HRM techniques. PCR reactions were carried out in a 10-μl final volume using the LightCycler® 480 High Resolution Melting Master (Reference 04909631001; Roche Diagnostics) and contained 1X buffer, *Taq* polymerase, nucleotides and the ResoLight dye, and 10 ng of DNA. The primers and $MgCl_2$ were used at 0.25 μM and 2.5 mM, respectively, to detect *TNFAIP3* single-nucleotide polymorphisms.

The PCR program required a SYBR Green I filter (533 nm), and consisted of an initial denaturation activation step at 95˚C for 10 min, followed by a 45-cycle program (denaturation at 95˚C for 10 sec, annealing at 60˚C for 15 sec and elongation at 72˚C for 15 sec with reading of the fluorescence; acquisition mode: single). The melting program included three steps:

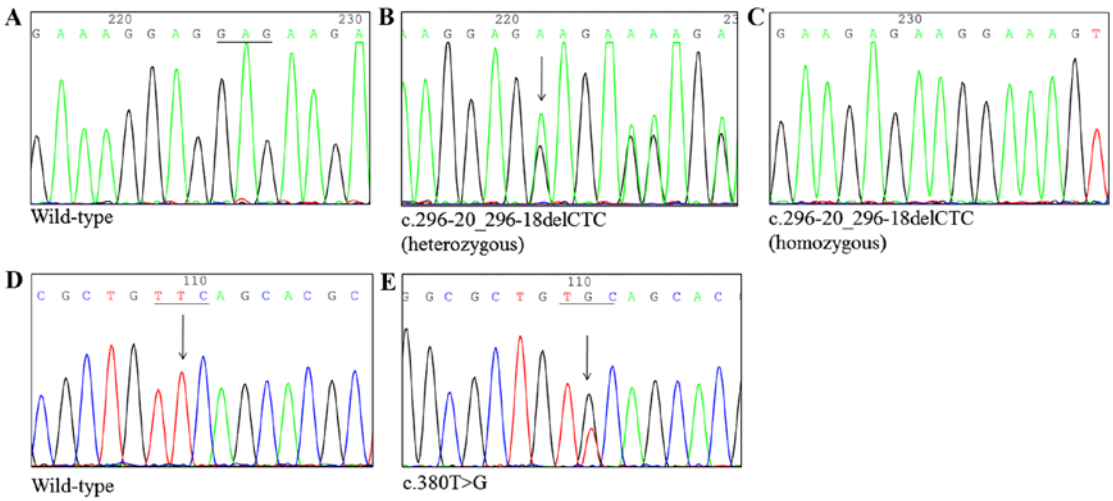

Figure 1. Direct sequencing of *TNFAIP3* exon 3. Sequencing results confirmed the (A and D) wild-type and the presence of the *TNFAIP3* exon 3 single-nucleotide polymorphisms: (B) c.296-20_296-18delCTC (heterozygous), (C) c.296-20_296-18delCTC (homozygous) and (E) c.380T>G.

denaturalization at 95˚C for 1 min, renaturation at 40˚C for 1 min and then melting with a continuous reading from 60˚C to 90˚C at 25 acquisitions per ˚C.

Direct DNA sequencing. After HRM analysis, the samples were purified using the PCR-M™ clean up system (Viogen, Sunnyvale, CA, USA). The sequence reaction was performed in a final volume of 10 μl, including 1 μl of the purified PCR product, 2.5 μM of one of the PCR primers, 2 μl of the ABI PRISM terminator cycle sequencing kit v3.1 (Applied Biosystems), and 2 μl of 5X sequencing buffer. The sequencing program followed a 25-cycle PCR program (denaturation at 96˚C for 10 sec, annealing at 50˚C for 5 sec, and elongation at 60˚C for 4 min). Sequence detection was performed using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) according to standard protocols.

Statistical analysis. Data analysis was performed using the SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). The Chi-square test was used to compare *TNFAIP3* genotype distributions between the case and control groups. Univariate unconditional logistic regression analyses were used to obtain odds ratios (OR) and corresponding 95% confidence intervals (CI). A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Screening of TNFAIP3 mutations in exons 2, 4, 5, 6 and 8. To identify *TNFAIP3* gene mutations in exons 2, 4, 5, 6 and 8, we designed primer pairs specific for each exon region (Table I). HRM analysis of exons 2, 4, 5, 6 and 8 showed no nucleotide changes in these regions (data not shown).

Screening of TNFAIP3 mutations in exon 3. We used one primer pair to identify *TNFAIP3* mutations in exon 3. The PCR amplification product contained multiple SNPs. Thus, this PCR primer was not suitable for HRM. Consequently, this exon was analyzed by direct sequencing. Fig. 1 shows that one SNP resulted in a change from thymine to guanine at position 380 (c.380T>G), and another caused a deletion of cytosine, thymine and cytosine (CTC) at position 296 (c.296-20_296-18delCTC). The c.380T>G change resulted in a Phe127Cys amino acid substitution. In addition, the c.380T>G nucleotide change was found in four subjects, for an incidence of 4.94%, while a three nucleotide deletion (c.296-20_296-18delCTC) was found in 80 subjects, for the highest incidence of 98.77%.

Screening of TNFAIP3 mutations in exon 7. We used four primer pairs (P7-1, P7-2, P7-3 and P7-4) to identify *TNFAIP3* mutations in exon 7. As shown in Fig. 2, three mutations resulted in a change from guanine to adenine at position 1081 (c.1081G>A), from cytosine to guanine at position 1398 (c.1398C>G), and from cytosine to thymine at position 1760 (c.1760C>T). These were confirmed by direct sequencing of the codons of interest. The c.1081G>A, c.1398C>G and c.1760C>T changes resulted in Glu361Lys, Ser466Arg, and Pro587Leu amino acid substitutions, respectively. Intriguingly, the mutation p.E361K has not been reported previously, which the PolyPhen-2 predicted as a benign mutation. In addition, mutations in exon 7 were found in three subjects, for an incidence of 3.7%.

Screening of TNFAIP3 mutations in exon 9. We used two primer pairs (P9-1 and P9-2) to identify *TNFAIP3* mutations in exon 9. One SNP resulted in a change from cytosine to thymine at position 2140 (c.2140C>T) (Fig. 3). This was confirmed by direct sequencing. The c.2140C>T change resulted in a Pro714Ser amino acid substitution. Intriguingly, the SNP p.P714S is similar to the amino acid change Pro714Ala, which has been reported previously (rs369155845). In addition, a c.2140C>T nucleotide change was found in one subject, for an incidence of 1.2%.

TNFAIP3 polymorphisms in normal controls. We recruited 50 healthy Taiwanese subjects from the China Medical University Hospital. DNA from the subjects was screened for *TNFAIP3* mutations. We identified five SNPs by HRM analysis, which were confirmed by direct DNA sequencing: c.296-20_296-18delCTC, c.296-15C>T, c.305A>G, c.380T>G and c.2140C>T (Fig. 4). In addition, the nucleotide changes

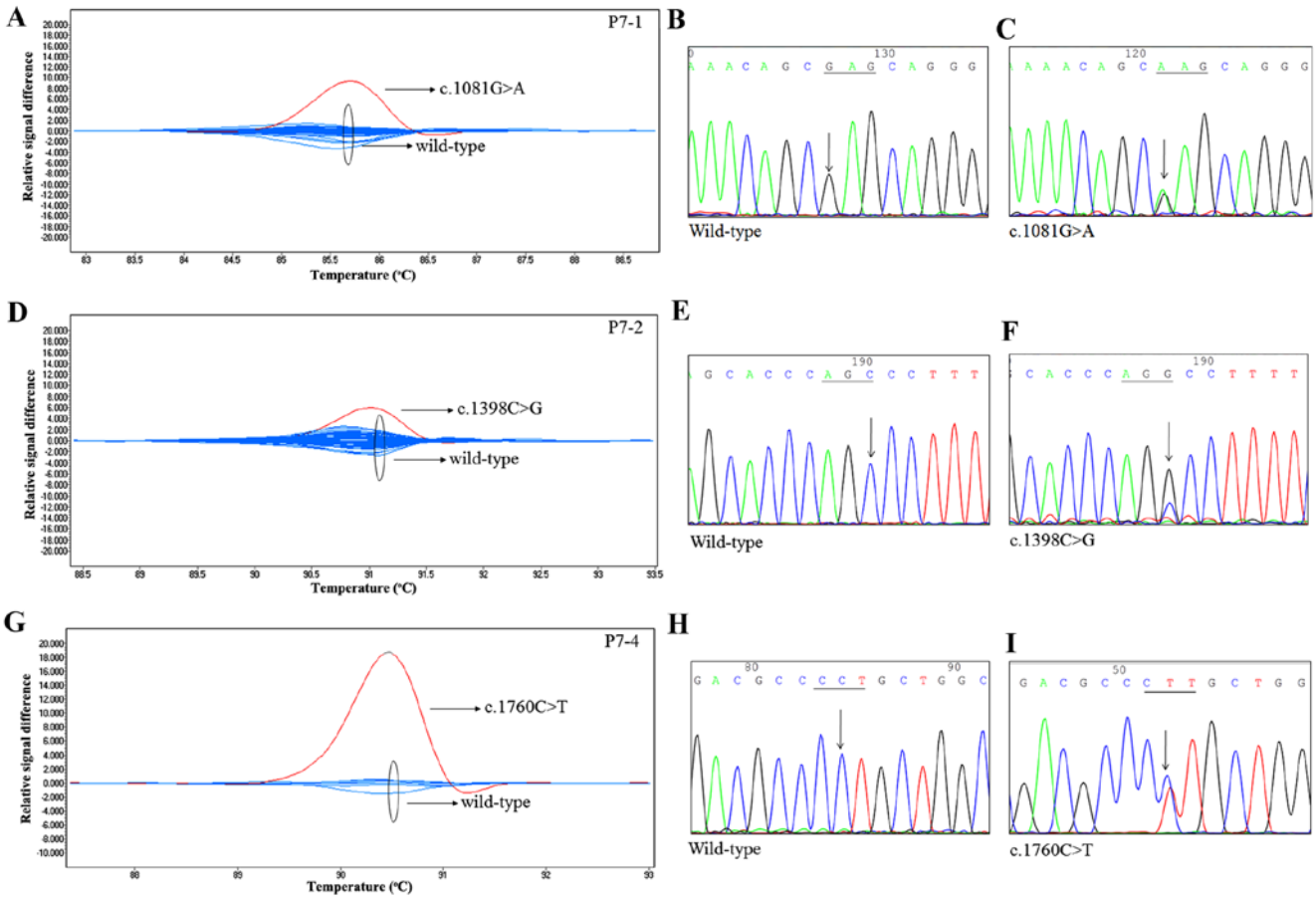

Figure 2. HRM assays for *TNFAIP3* in exon 7. (A) Mutation screening at exon 7 of the *TNFAIP3* gene using the primer pair P7-1. Sequencing results confirmed the (B) wild-type and the presence of the *TNFAIP3* exon 7 mutation (C) c.1081G>A. (D) Mutation screening at exon 7 of the *TNFAIP3* gene using primer pairs P7-2. Sequencing confirmed the (E) wild-type and the presence of the *TNFAIP3* exon 7 mutation (F) c.1398C>G. (G) Mutation screening at exon 7 of the *TNFAIP3* gene using the primer pair P7-4. Sequencing results confirmed the (H) wild-type and the presence of the *TNFAIP3* exon 7 mutation (I) c.1760C>T.

Table II. Haplotype frequency of *TNFAIP3*_rs71670547, rs377482653, rs146534657 and rs2230926 among the OSCC patients.

Haplotype	Cases n (%)	Controls n (%)	OR	95% CI	P-value
del-C-A-T	149 (92)	88 (88)	1	Ref	
CTC-C-A-G	4 (2)	7 (7)	0.34	0.10-1.19	0.0771
CTC-C-A-T	9 (6)	3 (3)	1.77	0.47-6.72	0.3946
CTC-C-G-T	0 (0)	1 (1)			
del-T-A-T	0 (0)	1 (1)			

OR, odds ratio; CI, confidence interval.

with c.296-20_296-18delCTC, c.296-15C>T, c.305A>G, c.380T>G and c.2140C>T were found in 50, 1, 1, 7, and 1 subject, respectively, for incidences of 100, 2, 2, 14 and 2%, respectively.

Frequencies of the TNFAIP3 haplotype. The rs71670547, rs377482653, rs146534657 and rs2230926 haplotypes were analyzed to investigate susceptibility to OSCC. The haplotype CTC-C-A-G was marginally inversely associated with a high risk of OSCC (OR=0.34; 95% CI=0.10-1.19; P=0.077; Table II).

Discussion

Several *TNFAIP3* mutations involving various substitutions in multiple tumor types have been reported [Table III, extracted from the Catalogue of Somatic Mutations in the Cancer (COSMIC) Database]. Many studies have focused on *TNFAIP3* genetic variations in hematopoietic and lymphoid tissue. According to the COSMIC database, the *TNFAIP3* mutation in OSCC has been reported by only one study from the USA using whole-exome sequencing (20), and the only mutation (p.Y614C) identified in that study was not found in the Taiwanese population of our study. Conversely, we identified three mutations by HRM analysis (p.E361K, p.S466R and p.P587L) and confirmed them by direct sequencing. This discrepancy in results may reflect different lifestyle risk factors. For example, oral cancer patients from Taiwan are frequently exposed to betel-quid chewing, and the betel-quid used in Taiwan is different from that used in other countries.

Although the HRM method is a powerful screening tool, it has some limitations; one of which is that unexpected polymorphisms present in the mutation of interest may interfere with genotyping. Therefore, we designed amplicon lengths of 100-300 bp, the suggested ideal size for HRM analysis. Two polymorphisms in intron 2 (rs71670547 and rs377482653) and two in exon 3 (rs146534657 and rs2230926) caused difficul-

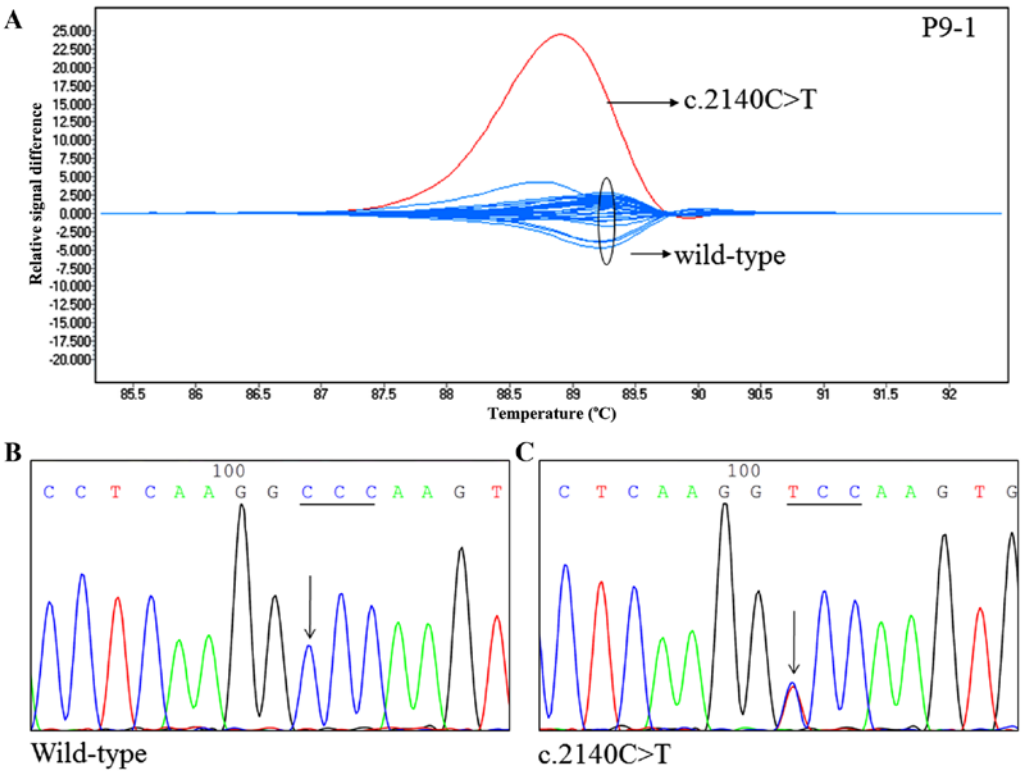

Figure 3. HRM assays for TNFAIP3 exon 9. (A) Normalized and temperature-shifted difference plots showing two melting profiles, the wild-type (WT) sample is blue, and the single nucleotide alteration is red. Sequencing results confirmed the (B) wild-type and the presence of the *TNFAIP3* exon 9 single-nucleotide polymorphism (C) c.2140C>T.

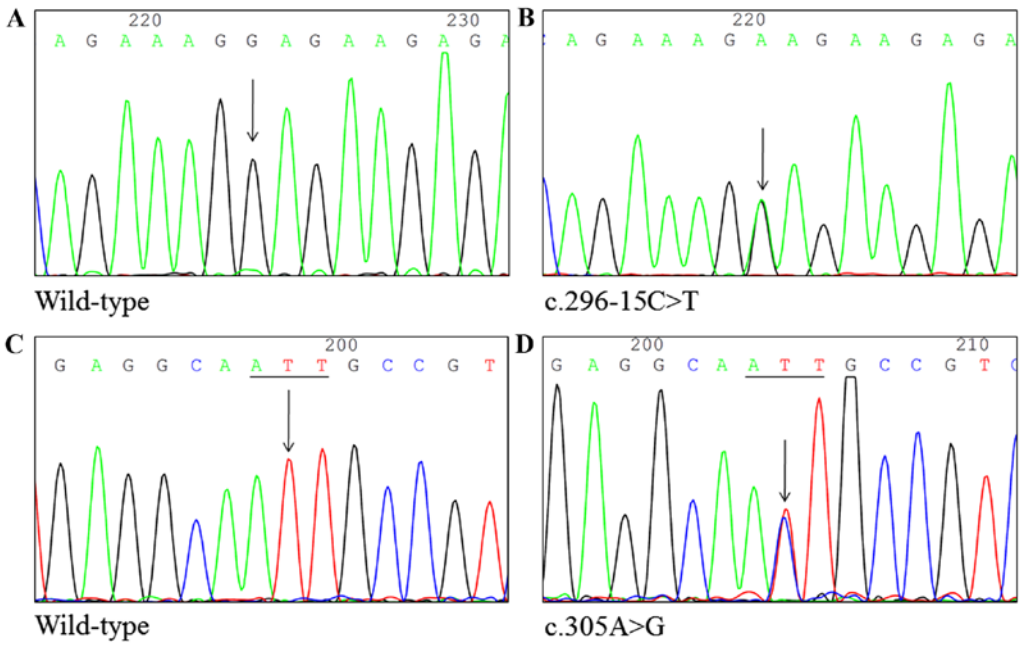

Figure 4. Application of HRM and direct sequencing for analysis of *TNFAIP3* single-nucleotide polymorphisms in 50 normal individuals. Sequencing results confirmed the (A and C) wild-type and the presence of the *TNFAIP3* exon 3 single-nucleotide polymorphisms (B) c.296-15C>T, and (D) c.305A>G.

ties in the HRM analysis; these polymorphisms could not be differentiated using a melting curve. To avoid this, the designed primers should flank the exon or intron as closely as possible. When an SNP is close to the exon or intron boundary, the primer can be placed over the SNP and a mismatched base with no allelic preference can be introduced at the SNP position (21). If the amplicon length is increased, the wild-type and heterozygote curves become smaller and are more difficult to distinguish. The HRM method is unable to detect mutations encompassing an entire exon or deletions of entire genes and exons.

Table III. *TNFAIP3* gene mutations in a variety of tumor types, extracted from the COSMIC database.

Tumor type	*TNFAIP3* Mut	All samples	%	Substitution - Nonsense	Substitution - Missense	Substitution - Synonymous	Insertion - Frameshift	Deletion - Inframe	Deletion - Frameshift	Complex	NonStop extension	Whole gene deletion	Unknown
Bone	1	75	1.33		1								
Breast	8	1,390	0.58	1	2	5							
Central nervous system	1	1,212	0.08			1							
Endometrium	3	505	0.59		1	1			1				
Haematopoietic and lymphoid	200	2,720	7.35	46	32	4	34	1	62	2	1	25	18
Kidney	1	961	0.1			1							
Large intestine	64	830	7.71	3	35	25	1		2				
Liver	6	942	0.64		5								1
Lung	21	1,505	1.4		15	6							
NS	1	240	0.42		1								
Oesophagus	1	261	0.38		1								
Ovary	4	823	0.49		4								
Pancreas	2	913	0.22		1								
Prostate	2	503	0.4		2								
Skin	1	334	0.3		1								
Stomach	2	47	4.26		2								
Thyroid	3	32	9.38		2	1							
Upper aerodigestive tract	1	244	0.41		1								
Urinary tract	3	366	0.82		2	1							
Our present study	3	81	3.7		3								

Mice with the A20 deletion in intestinal epithelial cells (IECs), B cells, myeloid cells, dendritic cells (DCs) and keratinocytes have been investigated extensively. Specific A20 deletions in B cells exhibit enhanced B-cell proliferation and survival and autoantibody production (22-24). Mice with the A20 deletion in all cells of myeloid origin develop spontaneous polyarthritis with the production of type II collagen autoantibodies and inflammatory cytokines in serum (25). In addition, mice with a DC-specific A20 deletion developed either SLE-like symptoms or human inflammatory bowel disease in independent studies (26,27). Furthermore, mice with A20-deficient IECs are highly sensitive to dextran sodium sulfate-induced colitis and TNF due to IEC apoptosis and loss of barrier integrity (28). Finally, A20 expression was significantly decreased in human colorectal cancer samples compared with adjacent non-neoplastic mucosa (29), and mice with A20-deficient keratinocytes displayed keratinocyte hyperproliferation and ectodermal organ abnormalities (30). Thus, conditional gene targeting studies have demonstrated an important role for A20 in controlling tissue homeostasis.

In conclusion, the HRM DNA screening method provides a reliable, accurate, and rapid method of identifying *TNFAIP3* mutations for the clinical diagnosis of cancer.

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