

Detection of an activating c-kit mutation by real-time PCR in patients with anaphylaxis

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Abstract

A well-characterised gain-of-function point mutation within exon 17 of the *c-kit* proto-oncogene known as Asp816Val is present in patients with mastocytosis. Activation of mast cells through this receptor primes them for IgE-dependent activation, and patients with mastocytosis are at increased risk of anaphylaxis. We hypothesised that the Asp816Val mutation is associated with a history of anaphylaxis in the general population.

A mismatch amplification real-time PCR assay was developed and validated to test for the Asp816Val mutation. Subjects were recruited to four subject groups: normal non-atopics, atopics without anaphylaxis, food-induced anaphylactics and non-food anaphylactics. Blood samples collected from forty subjects were tested for the presence of Asp816Val.

Thirteen subjects were found to carry the mutation; normals (2/9), atopics (2/10), food anaphylactics (5/11) and non-food anaphylactics (4/10). Statistical analysis of the data determined that there was no significant difference between the numbers of subjects found to carry the Asp816Val mutation in each of the groups although a trend towards an increased occurrence in anaphylactics was observed. In summary, the hypothesis that the presence of the Asp816Val mutation is linked to the occurrence of anaphylaxis was not supported, but interestingly, we have shown for the first time Asp816Val may occur more frequently than previously reported within the general population.

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1. Introduction

Mast cells originate from progenitor cells in the bone marrow, circulate as undifferentiated mononu-

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clear cells in the peripheral circulation and subsequently mature under local influences following migration into tissue. Mast cells are widely distributed throughout the body in both connective tissue and at mucosal surfaces. They form a heterogeneous population of cells which have been implicated in allergic diseases, host defence, tissue remodelling, wound repair, pathological fibrosis, arthritis and tumour angiogenesis [1].

Anaphylaxis is a severe life threatening reaction caused by the acute release of preformed and newly generated autacoid mediators from mast cells [2]. It is estimated that from 1.2 to 15% of the population are at risk of an event [3]. Principle causes include hymenoptera stings, drugs such as penicillin, various foods, radiocontrast media and exercise. In addition, 15–30% of cases are designated ‘idiopathic’ with no precipitating factor being identified [4,5].

Mast cells require stem cell factor (SCF) to survive and proliferate. The SCF receptor, CD117, is encoded by the protooncogene, *c-kit*. CD117 dimerization and activation is dependent on exogenous SCF. However, mutations to *c-kit* have been identified which result in constitutive receptor signalling via endogenous tyrosine kinase activity [6,7]. Activating mutations occurring throughout the *c-kit* coding sequence have been reported in several proliferative disorders (reviewed in [8]). More recently, Asp816Val has been detected within samples taken from patients with idiopathic anaphylaxis [9]. The potential role of Asp816Val in other forms of anaphylaxis is unknown.

Investigations into the role of *c-kit* mutations in mast cell disease have been hampered by the absence of mature mast cells in blood. Analysis of the Asp816Val mutation is further complicated by the fact that all published data show this mutation to be somatic, and therefore not present within all cells. Recent publications have presented a method to analyse somatic *c-kit* mutations by analysing cells arising from a common progenitor which carry the mutation but do not express CD117 [10,11]. The method relies on the use of RT-PCR to amplify relatively small amounts of mutated *c-kit* mRNA.

The aim of the current investigation was to develop a rapid test to distinguish trace amounts of Asp816Val sequence even in the presence of much greater levels of wild type (WT) sequence. Real-time PCR (TaqManTM) technology permits gel-free prod-

uct detection throughout amplification using a dual-labelled fluorogenic sequence-specific probe located within the PCR primers. Before amplification, the quencher dye absorbs the fluorescence from the reporter dye. During amplification the 5′ nuclease activity of Taq cleaves the probe, separating the dyes and an increase in fluorescence is recorded. Comparison of this increase with the fluorescence of a passive internal reference dye provides information about the PCR cycle at which ‘threshold’ fluorescence is reached. A large amount of data are generated in a shorter time period than traditional PCR, in a 96-well format which is ideally suited to high throughput analysis of replicate samples with different primer pairs.

We present a real-time PCR method using mismatched primers designed to preferentially amplify Asp816Val sequence within a WT/mutant mix. The developed method was used to test for the presence of Asp816Val in subjects with food and non-food induced anaphylaxis as well as a control population of normal and non-anaphylactic atopic individuals. In addition, this study presents, for the first time, data for the occurrence of Asp816Val in normal individuals.

2. Materials and methods

2.1. Cloning *c-kit* coding sequence

Primers were designed based upon the published *c-kit* sequence (Genbank accession number XO6182). The *c-kit* coding sequence was amplified from human lung cDNA (Origene Technologies Inc., Cambridge Bioscience, UK). Amplified product was subsequently cloned into the mammalian expression vector, pGENIRESneoT4, which had a T4 transcription terminator inserted upstream of the CMV promoter, preventing transcriptional read-through of the coding sequence in *Escherichia coli*. An additional pGenIRESneo-*c-kit* construct containing the Asp816Val mutation was created using the QuikChangeTM site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands). The sequence of constructs was determined, and confirmed that the correct sequences were present and additionally showed the constructs to be GNNK-splice variants [12]. Constructs were transfected into *E. coli* SURE cells (Stratagene) and purified

plasmid DNA subsequently isolated using GibcoConcert Maxipreps (Invitrogen Ltd., Paisley, UK).

2.2. *c-kit* expressing cell lines

Human embryonic kidney cell line 293T (HEK) cells (American Type Culture Collection; LGC Promochem, Middlesex, UK) were transfected with *c-kit* construct or a green fluorescent protein (GFP)-expressing control pcDNA construct. Cells were transfected using DMRIE-C (Invitrogen) according to manufacturers instructions. Transfected cells were cultured in G418-supplemented medium (0.8 µg/ml) for 4 weeks to produce stable cell lines. Genomic DNA was extracted from 5×10^6 HEK-WT and HEK-Asp816Val cells using the Wizard Genomic DNA system (Promega UK, Southampton, UK). Published PCR primers [10] (forward 5'-aaaggagatctgtgagaataggctc-3', reverse 5'-agctcccaaagaaaatcccatagg-3') were used to amplify a 373 base pair (bp) region of *c-kit* exon 17. PCR products were sequenced by the Protein and Nucleic Acid Chemistry Laboratory sequencing service, University of Leicester, and sequencing data are shown in Fig. 1a and b.

2.3. Western blots to detect CD117

CD117 was immunoprecipitated from stable cell lines using 25 µg rabbit anti-human CD117 agarose-conjugated polyclonal antibody C19 (Autogen Bioclear, UK, Wiltshire, UK). Samples were electrophoresed on 4–12% gradient ready gels (Biorad Laboratories Ltd., Herts, UK) and analysed for the presence of CD117 using either goat anti-human CD117 polyclonal antibody (R&D Systems, Abingdon, Oxon, UK) or mouse anti-human phosphotyrosine monoclonal antibody (PY99; Autogen Bioclear UK). Bands were detected using an enhanced chemiluminescence system detection kit (Amersham International, Maidstone, Kent, UK) according to manufacturer's instructions, and are shown in Fig. 1c.

2.4. RNA extraction and cDNA synthesis from HEK cell lines

Total RNA was extracted from 5×10^6 cells for cell lines HEK-WT and HEK-Asp816 using the SV Total RNA isolation System (Promega). cDNA was synthe-

sised from 700 ng total RNA using the Superscript First Strand cDNA synthesis system (Invitrogen). Each real-time PCR reaction contained 5 µl of a 1:20 dilution of cDNA.

2.5. Real-time PCR method development

Mismatched forward primers [13] were designed to preferentially amplify Asp816Val sequence. The use of a mismatched forward primer with a 2 bp mismatch to the unwanted (wild type) allele at the 3' position but only a 1 bp mismatch to the allele of interest (Asp816Val) permits amplification of the allele of interest whilst minimising amplification of the unwanted allele. A forward primer with 100% homology to WT sequence was designed to be used as an internal reference standard to confirm RNA quality and permit comparison between samples. Primers and probe were designed using Primer Express software (Applied Biosystems, Cheshire, UK) to fulfil TaqManTM criteria. The probe was 5' labelled with 6-carboxyfluorescein (FAM) and 3' labelled with the fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA). Six Asp816Val-specific forward primers and four reverse primers were selected for testing. Primers and probe were synthesised by Sigma Genosys (Sigma Genosys Ltd., Cambridge, UK). Primer sequences are shown in Table 1.

Amplification reactions (25 µl) were prepared using Core Reagents (Applied Biosystems), and Axygen

Table 1
Primer and probe sequences

Primer name	Sequence
Forward WT	ttgtgatttgggtctagccagaga
Forward mutant 1a (F1a)	agatttggatttgggtctagccagact
Forward mutant 2a (F2a)	agatttggatttgggtctagccagaat
Forward mutant 3a (F3a)	agatttggatttgggtctagccagatt
Forward mutant 1b (F1b)	ttgtgatttgggtctagccagact
Forward mutant 2b (F2b)	ttgtgatttgggtctagccagaat
Forward mutant 3b (F3b)	ttgtgatttgggtctagccagatt
Reverse 1	gtgccatccacttcacaggtag
Reverse 2	ttcagggtgccatccacttca
Reverse 3	ctttcagggtgccatccacttc
Reverse 4	tgcttcagggtgccatcca
Probe	tcgagcgtttccttaaccataatagaatca

Differences between forward primers, which confer Asp816Val specificity are shown in bold.

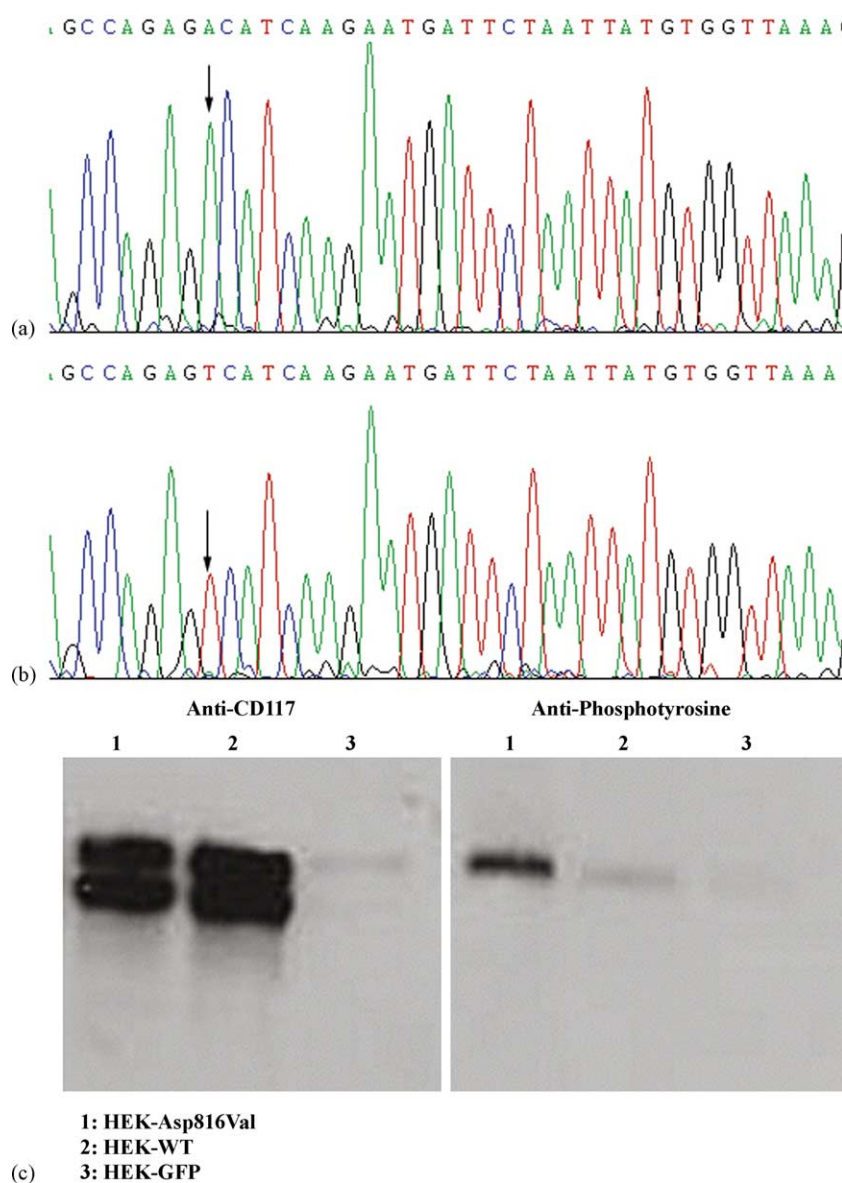


Fig. 1. Confirmation of cell line expression of transfected c-kit sequence. (a) Genomic DNA sequencing results for cell line HEK-WT. (b) Genomic DNA sequencing results for cell line HEK-Asp816Val. (c) Detection of CD117 and phosphorylated CD117 by Western blotting.

(Thistle Scientific Ltd., Glasgow, UK) 96-well semi-skirted plates. Final concentrations of 900 nM of each primer and 200 nM probe were used. Reactions were performed on an ABI Prism 7700 with the following thermal cycling protocol: 50 °C for 2 min, 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.6. Subject identification and recruitment

Subjects were recruited at Glenfield General Hospital, Leicester, according to defined criteria. Ethical consent for the investigation was granted by Leicestershire Research Ethics Committee and all patients provided written informed consent. Eligibil-

ity was confirmed from clinical records and by detailed interviews followed by skin prick testing (SPT) or IgE testing. Forty-two subjects were initially recruited to four groups: normals, non-anaphylactic atopics, food anaphylactics and non-food anaphylactics. All subjects with a history of anaphylaxis had a documented history of at least one severe reaction (hypotension, bronchospasm or glottal oedema), and had been issued with injectable adrenaline for emergency use. SPT was performed using ALK Abello reagents (Alk-Abello (UK) Ltd., Berkshire, UK), with a panel of 23 common allergens. Wheals greater than 3 mm diameter after 15 min incubation were considered positive. Serum samples collected from anaphylactic subjects were tested for total and specific IgE using the UniCAP system (Pharmacia & Upjohn, Stockholm, Sweden). Results were reported in KU/l and values above 0.35 KU/l were considered positive.

2.7. RNA extraction from blood samples

Blood samples (5 ml) were collected into heparinised tubes. A mixed population of T cells, B cells and monocytes was isolated from each blood sample by the addition of 100 µl each CD3, CD14 and CD19 Dynabeads (Dynal Biotech UK, Wirral, Cheshire, UK), following a modified version of a published protocol [10]. Total RNA was then extracted from the mixed cell population using the SV total RNA isolation system (Promega). Total RNA was stored immediately in aliquots at -70°C . cDNA was subsequently synthesised as required from a minimum of 75 ng total RNA using the Superscript First Strand cDNA synthesis system. Each real-time PCR reaction contained 5 µl of a 1:2 dilution of cDNA.

2.8. Real-time PCR using subject samples

TaqMan PCR experiments were performed in duplicate on at least two separate occasions for each subject with both forward primers (FWT and F1b), the common reverse primer Rev 1 and probe. Individual replicate results were collected as the threshold cycle number (Ct) at which fluorescence significantly exceeded that of an internal reference dye.

3. Results

3.1. Validation of HEK-Asp816Val and HEK-WT cell lines

The sequence analysis of genomic DNA extracted from HEK-WT and HEK-Asp816Val cell lines confirmed that the transfected mutations had been stably incorporated into the genome. Sequence data (Fig. 1a and b) shows a single bp difference between WT and mutant. Cells lysates were also analysed by Western blotting. CD117 was detected as a doublet comprising a 145 kDa mature form and a 122 kDa immature form. CD117 was also immunoprecipitated from the three cell lines and Western blots are shown in Fig. 1c. The doublet was present in HEK-Asp816Val and HEK-WT but not the control cell line HEK-GFP, confirming that the parent cell line did not constitutively express CD117. When a phosphorylation-specific antibody was used, the mature 145 kDa band was detected in the HEK-Asp816Val cell line lysate, which expressed the gain-of-function mutation, but not in the other lysates.

3.2. Real time PCR method development and validation

A real-time PCR method was developed and tested to determine its ability to distinguish between Asp816Val mutant and WT. Six mutant-specific forward primers were designed and tested. A forward primer with 100% homology to WT sequence was also used as an internal reference standard to confirm the presence of good quality c-kit cDNA and allow comparison between samples. Four reverse primers were also tested. Two variables were considered when designing Asp816Val-specific forward primers. For the forward primer to differ in the penultimate position from the normal base (G), the penultimate base of the primer could be A, C, or T. Three versions of the same primer (F1a, F2a and F3a) were designed incorporating A, C or T as the penultimate base, respectively. In addition, we investigated the effect of primer length upon allele specificity, by creating a second set of forward primers which were identical to the first set at the 3' end but were truncated by four bases at the 5' end to a total length of 24 bases (F1b, F2b, F3b) instead of 28 bases.

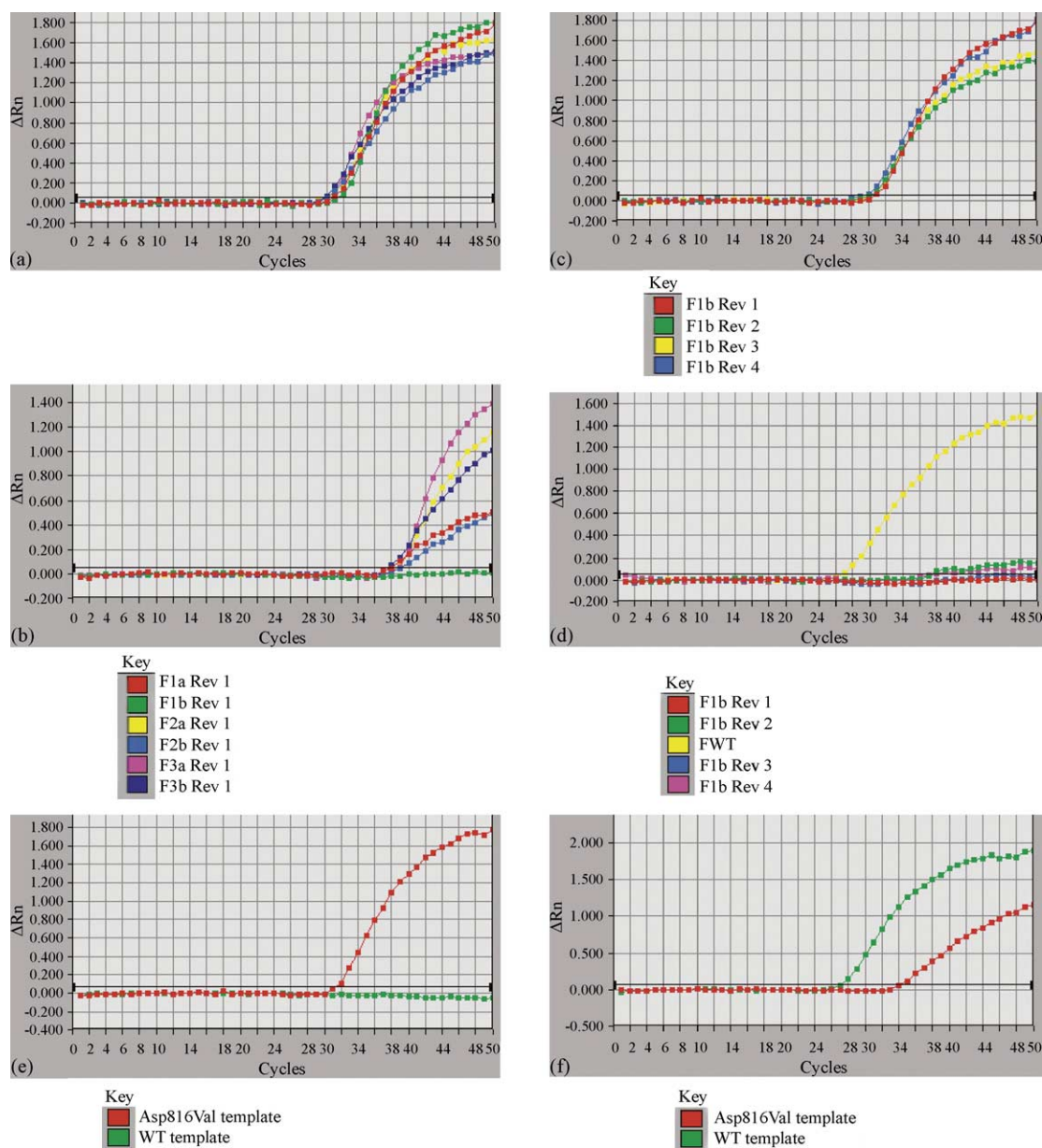


Fig. 2. Optimisation of the real-time PCR assay. The mutant specificity of six forward primers was tested using (a) Asp816Val template and (b) WT template. The effect of four reverse primers on the mutant specificity of the selected forward primer F1b was tested using (c) Asp816Val template and (d) WT template. Finally, the difference in amplification efficiency between the Asp816Val and WT templates was tested using the mutant specific primer F1b (e) and the positive control primer FWT (f).

To select the most appropriate primer pairs, experiments were conducted using cDNA prepared from RNA extracted from the two control cell lines HEK-WT and HEK-Asp816Val. The six mutant-specific forward primers were tested in combination with reverse primer 1 (Fig. 2a and b). Variations incorporated into the six primers did not have any effect upon amplification efficiency from Asp816Val template (Fig. 2a) but had a dramatic effect upon the amplification efficiency from WT template. Primers with A in the penultimate position (F1a, F1b) were most specific and those with T (F3a, F3b) least specific. However, the mismatched penultimate base alone was not sufficient to confer complete allele specificity, as only the shorter form of the primer (F1b) was found to exclusively amplify Asp816Val template but not WT template. For primer pairs F2a/2b and F3a/3b, although the primers were not exclusive for the mutant allele, improved specificity was observed with the truncated form of the primer. Primer F1b was therefore selected as an appropriate mutant-specific primer for use in future experiments.

To ensure optimal sensitivity, four reverse primers were also tested with primer F1b (Fig. 2c and d). Little difference in efficiency was detected with the four different primers, therefore primer Rev 1 was selected for

use in future experiments. A direct comparison of amplification using both WT and mutant templates with F1b/Rev 1 and FWT/Rev 1 was performed (Fig. 2e and f). F1b/Rev 1 exclusively amplified Asp816Val mutant template while FWT/Rev 1 preferentially amplified WT template but could also amplify from mutant template, with a 7 Ct unit reduction in efficiency.

The ability of primer F1b to distinguish between WT and Asp816Val mutant template was tested by mixing HEK-Asp816Val cDNA with HEK-WT cDNA prior to amplification. Mutant:WT mixtures from 1:10 to 1:200 were tested, and representative amplification plots are shown in Fig. 3. Each condition was tested in duplicate on two occasions, and the average Ct and variance between replicates are shown in Table 2. These data show that, as the proportion of WT template increases, the slope of the amplification curve flattens, Ct values increase and the degree of variance between replicate samples increases.

3.3. Using the real-time PCR method to test subject samples

Forty-two subjects were initially recruited for the investigation; one normal control and one non-food

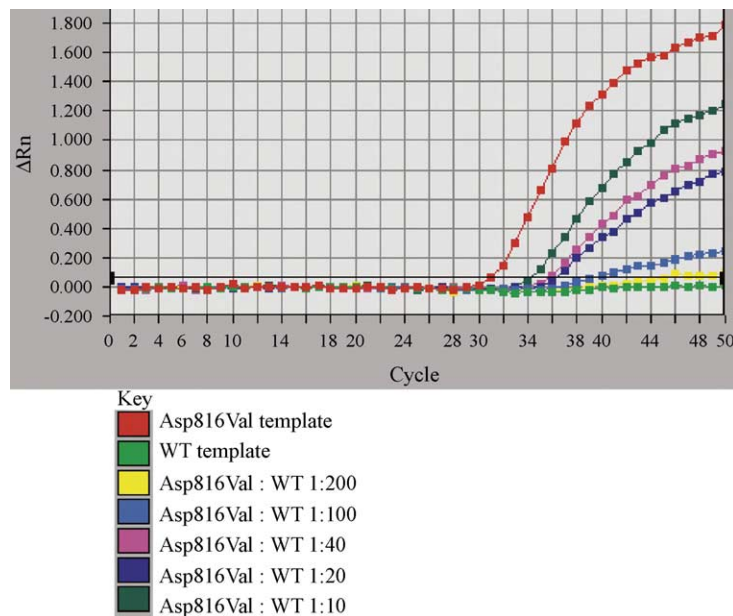


Fig. 3. Determination of the limit of detection of Asp816Val template in the presence of excess amounts of WT template using the real-time PCR assay.

Table 2
Limit of detection for Asp816Val F1b/Rev 1 primers and probe

	Average Ct	S.D.
WT	50	0
Asp816Val	32.42	0.79
Mutant:WT 1:10	35.8	0.80
Mutant:WT 1:20	35.91	2.04
Mutant:WT 1:40	36.49	3.27
Mutant:WT 1:100	44.45	4.58
Mutant:WT 1:200	47.44 ^a	5.13

^a Ct values above 45.0 are considered to be negative.

anaphylactic subject were subsequently removed from the investigation following disclosure of immune-cell mediated disorders. The clinical status of the remaining forty subjects is shown in Table 3. In all cases, FWT/Rev 1 was used as a positive control to confirm cDNA quality, and F1b/Rev 1 to test for the presence of Asp816Val. Representative amplification plots from eight subjects are shown in Fig. 4.

Amplification using the positive control primer pair FWT/Rev 1 was used to investigate the variance between subject groups. Average Ct values for the four subject groups are shown in Table 4a. Logistic regression using a Generalized Linear Model with binomial errors and logit link found there to be no significant difference between the four subject groups ($P=0.088$) despite a trend towards higher Ct values in the food anaphylactic group. All statistical analyses were performed using Genstat® (VSN International Ltd., Hemel Hempstead, Herts, UK).

Statistical analysis of replicate Ct values obtained using the F1b/Rev primer pair determined that average Ct values of 45.0 or less could be considered positive for the Asp816Val mutation. Thirteen subjects out of 40 were found to have the Asp816Val mutation at a consistently detectable level (Table 4b); this correlates to 21% (4/19) of the controls (normals/atopics) compared with 43% (9/21) of anaphylactics. Both atopic subjects shown to be positive for the mutation (subjects 23 and 24) were found during recruitment to have skin prick reactivity to food allergens. There was no clinical evidence to distinguish the mutation-positive normals (subjects 3 and 35). In group 4 (non-food anaphylactics), three subjects were allergic to wasp venom and the remaining seven subjects suffered from idiopathic anaphylaxis. Out of these subjects, four idiopathic anaphylactics were found to be positive for the mutation

(4/7) whilst none of the three wasp anaphylactics tested positive. There was no gender linkage with the occurrence of the mutation; the study population comprised 61% females and 39% males. The sub-population testing positive for the mutation comprised 64% females, 36% males. Logistic regression analysis found there to be no significant difference between the presence of Asp816Val in the four groups ($P=0.93$).

4. Discussion

We have developed a novel real-time PCR method for the rapid detection of the Asp816Val c-kit gain-of-function mutation. We present evidence that this mutation occurs within the general population, and is not specifically associated with a history of anaphylaxis.

When designing the current investigation it seemed likely that, if Asp816Val mutant template were present within samples, it would represent an extremely small proportion of the total c-kit template. Any detection system would therefore need to be sufficiently sensitive to differentiate between mutant and WT template in the presence of a large excess of WT template. The analysis of the Asp816Val c-kit mutation has been reported previously using standard RT-PCR amplification of mRNA template isolated from peripheral blood cells and subsequent sequencing of the product [10]. In a pilot study (data not presented), we prepared mixtures of known amounts of WT and Asp816Val mutant template to be used as template for PCR amplification and sequencing. We observed preferential amplification of WT template such that with 90% mutant template and 10% WT, only WT sequence was detected. We concluded that we were unable to detect Asp816Val mutant c-kit in the presence of WT template. Allele-specific primers which differed at only the ultimate 3' base were also unable to distinguish between mutant and WT template by traditional PCR amplification followed by gel electrophoresis to visualise the amplicon. It was therefore decided to utilise real-time (TaqManTM) technology in combination with mismatched primers to develop a high throughput, specific and informative assay.

Although real-time PCR is a sensitive technique, and the results of the current investigation do reveal the presence of Asp816Val mutant template in a mixed population of blood cells, Ct values in the range 30–45

Table 3
Clinical status of subject group

Subject	Group	Skin prick tests	Total IgE (KU/L)	Specific IgE (KU/L)/mast cell tryptase (µg/L)	Clinical history
3	Normal	Negative	<2	ND	NAS. Has child with anaphylaxis
6	Normal	Negative	51.1	ND	NAS
7	Normal	Negative	ND	ND	NAS
8	Normal	Negative	15.2	ND	NAS
9	Normal	Negative	ND	ND	NAS
13	Normal	Negative	13.6	ND	NAS
16	Normal	Negative	ND	ND	NAS
35	Normal	Negative	ND	ND	NAS
42	Normal	Negative	ND	ND	NAS
01	Atopic	Grass, HDM, cat, dog, rabbit, horse	ND	ND	Asthma, seasonal rhinitis
02	Atopic	Grass, HDM, ragwort	ND	ND	Asthma, seasonal rhinitis
10	Atopic	Grass, trees, birch	ND	ND	NAS
12	Atopic	Grass, cats, dogs	ND	ND	Asthma, seasonal rhinitis
14	Atopic	Grass, peanut	60.2	ND	Seasonal rhinitis
19	Atopic	Grass, trees, birch, HDM, cat, dog	ND	ND	Asthma, seasonal rhinitis
23	Atopic	Grass, HDM, shrimp	420	Crab 1.58, shrimp 1.78	NAS
24	Atopic	Grass, peanut, almond, mugwort	74.9	Peanut 0.87	Seasonal rhinitis
28	Atopic	Dogs, cats		ND	Asthma
33	Atopic	Grass, dogs	37.9	ND	Seasonal rhinitis
04	Food	Peanut, almond, hazelnut	98.9	Wheat 1.53, peanut 1.34, latex, 0.67	Anaphylaxis to nuts; asthma
20	Food	Peanut	19.3	Cod 3.27, peanut 0.38	Anaphylaxis to nuts and fish
26	Food	Grass, HDM, tree, cat, dog, horse, moulds (nuts ND)	158	Peanut 1.67	Asthma, eczema, rhinitis. Anaphylaxis to peanuts
29	Food	Cat, dog, HDM	>1000.0	Peanut 1.66, hazlenut 2.14, almond 0.82	Anaphylaxis to nuts
30	Food	Cat, rabbit, grass, peanut, almond, walnut, latex	345	Mixed nut screen negative	Anaphylaxis to nuts and latex
31	Food	Peanut, brazil nut	117	Mixed nut screen negative	Anaphylaxis to nuts
36	Food	Dog, grass, peanut	988	Shrimp 81.00	Anaphylaxis to prawns
37	Food	Dog, HDM, mould, horse, cat, feathers, grass,	1863	Brazil >100, peanut 25.3, hazelnut 16.7, almond 1.94, cashew 5.64, coconut 1.57, walnut 0.81	Anaphylaxis to nuts
38	Food	ND	161	Peanut 99.5, brazil 0.45, cashew 0.51	Anaphylaxis to nuts
39	Food	ND	72.4	Mixed nut screen 0.41, peas 0.52, soya bean 0.72, lentil 1.15	Anaphylaxis to nuts, baked beans, soya, peas
40	Food	Rabbit, peanut, brazil nut, almond, hazel, cashew	533	Brazil >100, hazlenut 16.7, soya bean 9.19, latex 1.35, egg white 2.93, egg yolk 1.14, walnut 0.81, peanut 0.51	Anaphylaxis to nuts, eggs, kiwi fruit; asthma
05	Nonfood	ND	55.8	Specific IgE negative	IA diagnosed 2002
11	Nonfood	Grass, HDM	79.4	Crab 0.36, shrimp 0.49; tryptase 5.3	IA diagnosed 2002

Table 3 (Continued)

Subject	Group	Skin prick tests	Total IgE (KU/L)	Specific IgE (KU/L)/mast cell tryptase ($\mu\text{g/L}$)	Clinical history
17	Nonfood	Cod	190.86	Specific IgE negative; tryptase 3.1	IA diagnosed 1999; no clinical reaction to cod
18	Nonfood	Negative	102	Specific IgE negative	IA diagnosed 2003
21	Nonfood	Grass	22.1	IgE ND; tryptase 10.0	IA diagnosed 2003
22	Nonfood	Negative (wasp ND)	113	Wasp (yellow jacket) 17.6	Anaphylaxis wasps
25	Nonfood	Negative	6.76	Specific IgE negative; tryptase 4.0	IA diagnosed 2003
27	Nonfood	Negative (wasp ND)	104	Wasp (yellow jacket) 0.43	Anaphylaxis hornets
34	Nonfood	Negative (wasp ND)	35.8	Wasp (yellow jacket) 1.72	Anaphylaxis wasps
41	Nonfood	Grass, horse, cat	79.16	Banana 1.7	IA diagnosed 2000; no clinical banana reaction

Mutation-positive subjects are shown in bold. Key: NAS: no allergic symptoms, ND: not done, IA: idiopathic anaphylaxis and HDM: house dust mite.

Table 4a

Analysis of data from real-time PCR using the positive control primer pair FWT/Rev 1

Group	Mean Ct	S.D.
Normal	33.62	2.58
Atopic	33.38	3.43
Food	35.98	3.18
Non-food	33.38	2.17

are much higher than would normally be expected from real-time analysis. This is not surprising, as a published investigation reported that as few as 3/65 cells analysed from one individual by single-cell PCR contained the Asp816Val mutation [11]. It seems probable that Asp816Val template detected in this investigation

Table 4b

Detection of Asp816Val in 13 subjects using the mutation-specific primer pair F1b/Rev 1

Subject	Group	F1b/Rev 1 average Ct
3	Normal	39.3
35	Normal	44.8
23	Atopic	38
24	Atopic	41.6
4	Food	42.2
20	Food	40.3
26	Food	41.1
31	Food	40.6
37	Food	39
5	Non-food	42.7
17	Non-food	42.6
18	Non-food	44.2
21	Non-food	44.4

may be derived from extremely small numbers of cells, with the vast majority of cells containing WT c-kit sequence. Another possible cause of assay inefficiency is the use of mismatched primers. Primer efficiency has in this case been sacrificed to ensure maximum specificity. Successful primer redesign to improve efficiency while retaining specificity may not be possible given the stringent criteria for TaqManTM primer design. Data presented in Fig. 3 show that the slope of the amplification curve decreases as the proportion of WT template increases; this suggests that apparent inefficiency may actually be due to competition between WT and mutant template. If this is the case then the selection of a more appropriate cell population to maximise the proportion of Asp816Val template present may improve Asp816Val detection.

The Asp816Val mutation has been detected previously in bone marrow biopsies or skin lesions taken from mastocytosis patients [8], but only rarely in total peripheral blood samples. The ability to identify the mutation from a blood sample was proposed as a possible marker of mastocytosis severity [14]. In 2000, a new technique was reported [10] which involved enrichment of specific leukocytes from peripheral blood samples prior to RNA extraction and sequence analysis. This permitted the detection of the Asp816Val mutation in at least one leukocyte cell type in 13/13 adult patients with indolent mastocytosis and urticaria pigmentosa. A further 3/5 patients with adult-onset indolent systemic mastocytosis were subsequently reported to possess the mutation [11]. In a recent publication [9], mast cell abnormalities, including the presence of codon 816 mu-

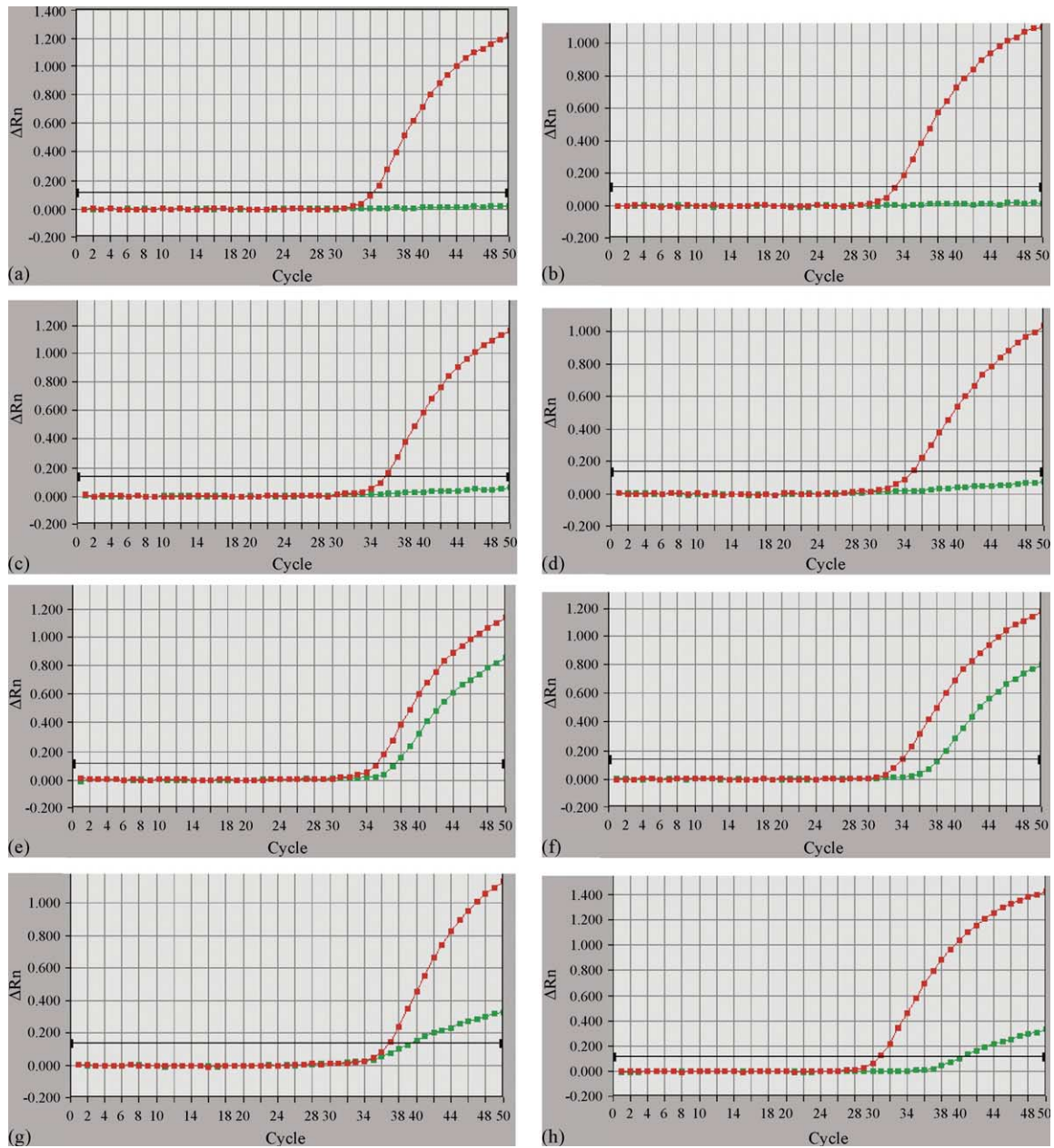


Fig. 4. Detection of the presence of Asp816Val in subjects by real-time PCR. Representative amplification plots are shown for eight subjects, with positive control primer FWT/Rev1 in dark grey (red online) and mutant-specific primer F1b/Rev1 in light grey (green online). (a) subject 12, (b) subject 18, (c) subject 28, (d) subject 42, (e) subject 26, (f) subject 23, (g) subject 3, (h) subject 31.

tations were reported for a group of five patients with idiopathic anaphylaxis. None of the three studies described [9–11] reported data for healthy normal subjects. This present study, to the best of our knowledge, reports the first investigation of normal subjects using the leukocyte enrichment technique.

It is, therefore, surprising to discover that a mutation linked to severe mast cell disease was present in 2/9 normal non-atopic subjects. The results of this investigation suggest that, in fact, Asp816Val may occur at a far higher frequency than previously reported. The relevance of this finding is unclear but it implies that the expression of Asp816Val is not sufficient on its own to cause either mastocytosis or allergic disease. If Asp816Val expression is linked to the occurrence of allergic disease then additional factors are likely to be required for disease expression. These could include the type of cell expressing the mutation, genetic predisposition to developing atopy and the interaction of complex environmental factors.

The enriched cell population studied in this investigation, comprising T cells, B cells and monocytes, was selected based upon a report describing Asp816Val detection in each of these cell types by single-cell PCR [11]. The c-kit proto-oncogene encodes the CD117 protein which is widely expressed on immature cells but, in mature cells, is restricted to mast cells. SCF ligand binds to CD117, resulting in phosphorylation, receptor dimerization and mast cell survival and proliferation. Expression of CD117 containing the Asp816Val mutation removes the requirement for receptor dimerization and permits ligand-independent mast cell survival. The detection of the Asp816Val mutation in non-mast cells, while interesting, is unlikely to be biologically relevant because CD117 is not expressed in those cell types. If, however, CD117 containing the Asp816Val mutation is expressed on the surface of mature mast cells in apparently normal individuals, it is likely to permit constitutive CD117 signalling and SCF-independent mast cell survival and proliferation. In this situation, Asp816Val expression could be a significant contributory factor to the severity of allergic disease. It would be desirable to repeat this investigation by extracting RNA directly from mature blood-derived mast cells. Mast cell culture from progenitors is, however, a complex and time-consuming procedure not suited to rapid diagnostic testing.

This was a small population study and so the identification of the mutation in normal subjects has limited its power to detect differences between the groups tested. However, one observation of particular interest was the occurrence of the Asp816Val mutation in 2/3 atopic patients with positive SPT results to food allergens (subjects 23 and 24). These subjects had no reported symptoms of food intolerance, which raises the question of whether they could be at risk of future systemic allergic reactions. Further investigation is required to fully evaluate the role of Asp816Val in food sensitivity using a much larger subject population.

In summary, a small difference was observed between the numbers of normal and atopic controls, and anaphylactics who tested positive for the presence of the Asp816Val mutation (21% and 43%, respectively). This difference was not statistically significant and does not support the hypothesis that the Asp816Val mutation is directly linked to the occurrence of anaphylaxis. However, there is no evidence to disprove an alternative hypothesis, that Asp816Val may be one of several genetic risk factors which increases the likelihood of developing severe allergic disease if the necessary environmental stimuli are encountered. In addition, a sensitive new technique has been developed which is highly specific for the Asp816Val mutation and may be applicable to clinical identification of Asp816Val in mast cells. Further investigation to address the choice of cell population and the sensitivity of the real-time assay are now required to confirm and expand upon the clinical impact of these preliminary findings.

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