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A Novel Flexible Multiplex Bead-based Assay for Detecting Germline *CDKN2A* and *CDK4* Variants in Melanoma-Prone Kindreds

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Abstract

Background—The presence of recurrent high-risk mutations in *CDKN2A* and *CDK4* among melanoma-prone kindreds suggests that a high-throughput, multiplex assay could serve as an effective initial screening tool. Moreover, with the emergence of new melanoma risk single nucleotide polymorphisms (SNPs) through genome-wide association studies, a flexible platform that can easily accommodate these new risk alleles is needed for more accurate genetic risk profiling. To this end, we have developed a novel melanoma-associated mutation detection method using a multiplex bead-based assay. This assay is suitable for high-throughput *CDKN2A* and *CDK4* genotyping and can be eventually adapted to multiple loci across various constituent populations.

Methods—Genomic DNA from a 1603 subjects (1005 in training set, 598 in validation set) were amplified by multiplex PCR using five primer sets followed by multiplex allele-specific primer extension for 39 different known germline variants. The products were then sorted on an xMAP™ (formerly Tag-It™) array and detected by use of the Luminex xMAP™ system. Genotypes were compared to previously-determined sequence data.

Results—In the Toronto training cohort, variants were detected in 145 samples, giving complete concordance between the bead assay and direct sequencing results. Analysis of the 598 samples from the GenoMEL validation set led to identification of 150/155 expected variants (96.77% concordance). Overall, the bead assay correctly genotyped 1540/1603 (96.07%) of all individuals

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in the study and 1540/1545 (99.68%) of individuals whose mutations were represented in the probe set. Out of a total of 62,512 SNP calls, 62,517 (99.99%) were correctly assigned.

Conclusions—In this initial evaluation, the multiplex bead-based assay for familial melanoma appears to be a highly accurate method for genotyping *CDKN2A* and *CDK4* variants.

Keywords

Melanoma; *CDKN2A*; *CDK4*; p14ARF; familial; high-throughput

Introduction

Cutaneous malignant melanoma (CMM) accounts for approximately 2% of new cancer cases diagnosed annually in North America. Of these cases 5–10% of individuals with CMM have an affected family member, indicative of inherited predisposition. The primary locus involved in familial CMM to date is *CDKN2A,* which encodes the cell cycle regulator protein p16, and when mutated accounts for 25–40% of familial CMM cases(Goldstein, et al., 2006; Goldstein, et al., 2007; Zuo, et al., 1996). The p16 protein is encoded by exons 1α, 2 and 3. An alternative transcript, comprising exons 1β, 2 and 3, encodes a protein, designated p14ARF because the exon 2 sequence is translated in an alternative reading frame relative to that of p16. Germline mutations in exon 1β of p14ARF have been detected in melanoma prone families(Harland, et al., 2005; Hewitt, et al., 2002; Rizos, et al., 2001a; Rizos, et al., 2001b). In addition, rare mutations in the gene encoding cyclin-dependent kinase 4 (*CDK4*)- the primary enzyme inhibited by p16 binding- have been identified in a small number of CMM kindreds worldwide(Goldstein, et al., 2006; Molven, et al., 2005; Soufir, et al., 1998; Zuo, et al., 1996).

The identification of these genes and their association with familial CMM has highlighted a need for flexible, cost effective, high-throughput genotyping methods, which can eventually be transferred to a diagnostic setting when melanoma risk profiling becomes clinically practical. In this report we describe the development and validation of a novel multiplex bead-based assay to detect CMM associated variants. This assay employs the xTAG™ (formerly Tag-It™) Microsphere-Based Universal Array Genotyping platform (Luminex Molecular Diagnostics, Toronto, Canada) originally described by Bortolin et al.(Bortolin, et al., 2004) in relation to thrombophilia.

The CMM-specific mutation detection assay described here can be readily modified to reflect certain population-specific mutations and to include any novel mutations or low-to-moderate risk alleles that may be shown to be associated with CMM. This study demonstrates the use of a microsphere-based array assay for the detection of 32 common *CDKN2A* variants, two *CDK4* mutations and five p14ARF variants among a large collection of familial melanoma samples from around the world.

Materials and Methods

Patient samples

A total of 1649 individual samples were genotyped. 1018 of these samples were accrued from March 1996 through May 2004 for *CDKN2A* research-based genetic testing through the Familial Melanoma Clinic at the Toronto-Sunnybrook Regional Cancer Centre (Toronto, Canada) and were from CMM affected patients, their unaffected relatives and spouses. All patients gave informed written consent to participate in this research study allowing a portion of their sample to be utilized for *CDKN2A* testing and the remainder to be banked for future studies. The remaining 628 samples were from international melanoma pedigrees

from the Melanoma Genetics Consortium (GenoMEL www.genomel.org). Ethical committee approval was obtained from all institutions involved. The GenoMEL samples included in the analysis are shown in Table 1. All assay development and initial validation were performed in Toronto. As part of the expanded Boston validation, the device was transferred to the Massachusetts General Hospital and recalibrated. All experiments in Boston were performed to the exact specification of the protocols delineated by Toronto except primer sets were re-synthesized and beads were purchased separately from the manufacturer.

DNA

For the Toronto subjects who were used in development of the assay, genomic DNA was isolated from whole blood with Qiagen QIAamp Blood Kits following the manufacturer's instructions. DNA samples were stored at −20°C until required. Before analysis, genomic samples were quantified spectrophotometrically by measuring absorbance at 260 nm, diluted to 5 ng/μl, and stored at 4°C. For the GenoMEL validation cases, sample derivation and DNA preparation were separately performed at each site; genetic analysis based on the GenoMEL material has been previously published(Goldstein, et al., 2006; Goldstein, et al., 2007).

DNA amplification

Twenty-five ng of genomic DNA samples were subjected to multiplex PCR amplifications which contained 1μl 10X PCR Buffer (Invitrogen), 0.3 μl 50mM Mg^{2+}, 1 μl dNTPs, 0.6 μl PCR primer mix (0.1 pmol each; IDT), 0.5 μl DMSO, 0.5 μl Platinum® *Taq* DNA Polymerase (Invitrogen) and ddH_2O to a final volume of 10 μl. Samples were amplified for i) *CDKN2A* exons 1α, 2, 3; and ii) *CDK4* exon 2 and p14ARF exon1β under the following cycling conditions: 95°C for 1 min, followed by 40 cycles of 95°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec and then an extension time of 72°C for 7 min. Samples were kept at 4°C until ready for use. A negative PCR control was included in each multiplex reaction. The primers and amplicon sizes for all PCR reactions are shown in Table S1. Multiplex PCR products were analyzed by gel electrophoresis to confirm the presence of discrete bands for each amplicon (data not shown). Since the 1–8dup8 change is a perfect repeat and silent to the allele-specific methodology, it was assayed by a PCR-gel based system and not by the bead analysis.

Amplicons were then subjected to Exonuclease I/Shrimp Alkaline Phosphatase (Exo/SAP; MBI Fermentas) treatment for 1 hour at 37°C to degrade any excess PCR oligonucleotides and inactivate any remaining nucleotides, particularly dCTP, as biotin-dCTP is incorporated during the primer extension reaction. Samples were Exo/SAP inactivated at 80°C for 15 min. The treated PCR products were then diluted 1:2 by the addition of 10 μl of ddH_2O.

Allele-Specific Primer Extension reaction

The microsphere-based assay was performed as described by Bortolin et al.(Bortolin, et al., 2004). Treated PCR products were used as the template in an Allele-Specific Primer Extension (ASPE) reaction, using SNP-specific oligonucleotide pairs, one terminating with the wild-type base, the other with the variant base (Table S2).

The ASPE sequence-specific oligos are tagged at the 5' end with a 24-mer universal tag sequence (Luminex Molecular Diagnostics (formerly TM Bioscience Corporation), Toronto, Canada). The ASPE reaction incorporates Biotin-labeled dCTP, in excess, which is used to quantify reaction products in the Luminex xMAP™ flow cytometer.

ASPE reactions were carried out with 1.25 μl Exo/SAP treated PCR product, 0.5 μl 10X PCR reaction buffer (Tsp; Invitrogen), 0.125 μl 50 mM Mg^{2+}, 0.1 μl 5μM dNTP-C (Amersham), 0.25 μl 5 μM biotin-dCTP (Invitrogen), 0.125 μl ASPE primer mix (1.25 pmol/μl each; IDT), 0.075 μl 5U/μl Tsp and ddH_2O to a final volume of 5 μl. The cycling conditions were as follows: 96°C for 2 min, followed by 40 cycles of 94°C for 30 sec, 54°C for 30sec and 74°C for 30sec. Samples were then stored at 4°C until ready for use.

A population of carboxylated microspheres (beads) tagged with complementary (anti-tag) sequences was then hybridized directly to the ASPE products by first denaturing the samples for 2 min at 95°C, and a reporter (streptavidin-conjugated phycoerythrin; SA-PE) was added to detect incorporated biotin.

The hybridized products were then run through the Luminex xMAP™ and classified on the basis of microsphere identification. The presence of each allele was quantified by SA-PE detection as described previously(Bortolin, et al., 2004). Figure 1 gives a schematic outline of the bead-based assay.

Statistical Analysis

The raw data, median fluorescent intensity (MFI), was collected for each bead population and base-lined by subtracting the value obtained for a PCR control sample (no DNA) from each sample value (Net MFI). Genotypes were determined from calculating the allelic ratios by using the ratio of Net wild-type or mutant MFI/sum of wild-type and mutant MFI for each SNP. This analysis could then determine wild-type, mutant, and heterozygous SNP genotype

DNA sequencing

Bidirectional DNA sequencing was performed on all 1018 samples using an MJ Base station as described previously(Hogg, et al., 2001) to compare sequences with the bead-based assay genotyping results. The 628 samples supplied by GenoMEL had previously been sequenced at source and at Leeds, as part of studies for other GenoMEL initiatives.

Results

Primary development of the multiplex bead-based assay was carried out on 1018 banked DNA samples from CMM affected individuals, unaffected relatives and spouses referred to the Familial Melanoma Clinic at the Toronto-Sunnybrook Regional Cancer Centre and for which the DNA material was successfully amplified. Since the 1–8dup8 mutation (N=13) is a perfect repeat and undetectable by the ASPE-based strategy, this variant was not incorporated into the bead assay. Thus, for the initial Toronto screen, 1005 samples (i.e. 1018-13) were blindly subjected to analysis for 39 variants (32 *CDKN2A* variants in one assay, two *CDK4* and five p14ARF splice site variants in another, Table S2). In total, 145/1005 (14.42%) samples were shown to harbor an identifiable *CDKN2A* variant, of which 65 (6.46%) samples carried the p.A148T polymorphism. Figure 2 depicts an example of a heterozygous p.T93P *CDKN2A* mutation with wild-type sequence at all other SNPs. There was 100% concordance between the bead-based assay and DNA sequencing; we in fact detected a *CDK4* p.R24C mutation that was previously undetected and subsequently confirmed by DNA sequencing. None of the p14ARF mutations were identified in the 1005 Toronto samples screened.

Our validation phase was then expanded to include 628 samples from CMM melanoma pedigrees supplied by the international melanoma genetics consortium, GenoMEL. The bead assay was performed blindly on each sample. A total of 30/628 (4.77%) DNA specimens failed to amplify. From the 598 scorable samples, 150/598 (25.08%) were found to harbor a

variant using the bead-based assay. When the sequence results were unblinded, 200/598 (33.44%) samples actually carried a variant. There were 45 mutations that could not have been detected based on assay design (seventeen 1–8dup8 mutations and 28 unprobed mutations, Table 1). For the 155 variants that were "eligible" for the assay, 150 were properly identified (150/155; 96.77%).

When the data from the training and validation sets were combined, 295/300 (98.33%) eligible mutations were successfully detected. However, since personal genotyping includes the accurate assignment of wildtype states, on an individual level, 1540/1603 (96.07%) of all available patient samples were correctly genotyped (i.e. variant(s) carrier vs. wildtype). This somewhat overestimates the error rate since 58 individuals had mutations not represented in the probe set. If these individuals were censored, the assay correctly genotyped 1540/1545 (99.68%) of the individuals with genotypes eligible for the assay.

Unlike sequencing, the bead assay performs 39 distinct SNP interrogations from 39 separate probe sets. Thus, it is also reasonable to measure the performance of the assay based on percentage of correct SNP assignments. For ineligible cases, where the mutations were not represented by one of the 39 probes, the assay was agnostic to the actual mutation but still made 39 correct sequence assignments at the individual SNPs. When considering SNP assignments in this study, a total of 62,517 SNPs were analyzed with only 5 incorrect calls. The bead-based assay is therefore 99.98% accurate at SNP scoring.

Four variants which had probe representation but were missed by the bead assay (false negatives) included two c.241_254del14 mutations, one splice site variant (sp3: c.IVS2+1G>T), and two p.A148T polymorphisms. One sample returned a false-positive result by being erroneously called as a p.G23D mutation when in actual fact the sample carried a mutation not included in the SNP panel. This false-positive result cannot be explained, as when we repeated the assay it appeared as wild-type. This was likely due to PCR product quality and/or quantity variations between the original and repeat runs. There were 28 variants among GenoMEL samples that were not included in the original assay (Table 1).

Discussion

A robust "lab-on-a-chip (LOC)" is an appealing notion in the emerging era of point-of-care diagnostics. Inherent to the fabrication of a genetic LOC is the need for a flexible platform that can respond to an enlarging repertoire of risk loci and the specific panel of founder changes embedded within certain populations. In this manuscript, we developed a novel multiplex bead-based assay, which can accurately genotype variants in the two established high-risk melanoma loci (i.e. *CDKN2A* and *CDK4*), and validated this assay on a cohort of familial melanoma patients. The fundamental rationale for developing such a system comes from the collective GenoMEL experience. A significant fraction of the 190 *CDKN2A* and *CDK4* mutation-bearing families reported by the consortium harbor recurrent germline mutations(Goldstein, et al., 2006); in fact, the 27 most prevalent variants account for over 80% of the detected mutations among the melanoma families ascertained by GenoMEL. The high frequency of founder changes observed among this cohort makes this an ideal collection to develop this novel mutation-interrogation platform. However, the assay was developed with a single set of testable variants in mind and is not expected to capture all possible *CDKN2A* mutations latent in the CMM population; thus our assay should be viewed only as a first generation design which can be readily expanded to include additional melanoma risk alleles.

A total of 1603 patient DNA samples were genotyped by both the Tag-It assay and DNA sequencing for exons 1α, 2 and 3 of the *CDKN2A* gene, exon 2 of the *CDK4* gene, and exon1β of p14ARF. With only 5 missed calls, the results between the bead-based assay and DNA sequencing were 98.33% in agreement by genotyping and 99.99% accurate by SNP calls. In terms of per sample cost, direct sequencing (forward and reverse) of the 5 exons (four *CDKN2A* and one *CDK4*; 10 separate reactions) costs approximately $20.00 at the MGH sequencing core ($2/sequence reaction) while bead analysis costs approximately $9.35 (25¢ for 70 primer sets + 13¢ per bead color × 70 bead colors). With further refinement, the economic advantage of bead assays could be further enhanced. In this initial proof-of-concept, the 39 variants can be found in 5 exons; however, if the 39 variants were in fact embedded in 39 separate exons, the cost of direct sequencing would be much higher. Although still early in terms of technical development, these results are quite encouraging for this novel bead-based assay.

In terms of assay development, the main technical advantages of this platform are its "plexibility" and flexibility. Both fluorescent-based and MALDI-driven SNP detection offer significantly lower multiplexing capabilities despite their flexibility of design. With newer systems, the bead-based assay can easily accommodate up to 500 SNPs in a single tube; thus, nearly all known variants of *CDKN2A*, *CDK4* and *ARF* can be incorporated into a single assay along with a host of low-to-moderate risk CMM alleles. Solid array systems (i.e. microarray "chips") present extraordinary capabilities in terms of parallel SNP analysis ($> 1\times10^6$ SNPs); however, there is little if any user input into the choice of SNPs or assay design unless a "custom chip" is manufactured. Unlike solid-phase microarrays, however, liquid bead arrays offer several unique advantages: the spherical substrate allows for a greater analytical surface area, the liquid phase mobility encourages more efficient mixing of cognate molecules and the encoded beads can be more readily manipulated. Emerging technologies that capitalize on microfluidics also hold promise for heightened throughput and flexibility(Derveaux, et al., 2008).

A major potential application of the bead-array assay is in SNP-based risk profiling. Over the past few years, a harvest of CMM disease-associated SNPs has been uncovered through large and robust genome-wide association studies(Udayakumar and Tsao, 2009). From a scientific perspective, follow up analysis of putative regions will need to be performed with greater SNP densities. As such, assays need to be rapidly developed for high-throughput analysis without sacrificing multiplexing capabilities. The bead assay is an ideal research platform for saturating these regions with additional SNP markers in hopes of refining the disease-associated intervals. As novel melanoma risk alleles are discovered, the single tube assay, which measures a single allele in a single reaction, will have to be replaced by array-based methods to be capable of analyzing multiple alleles in a single reaction. Thus, the bead assay can be configured to be a truly "personalized" risk predictor allowing SNP panel selection based on historical or phenotypic features.

Bead-based analytics have already been deployed in the clinical setting(Bortolin, et al., 2004) developed the xTAG™ (formerly Tag-It™) platform for the multiplex analysis of a panel of thrombophilia-associated SNPs. They reported 100% concordance between the xTAG™ assay and direct DNA sequencing data for 736 SNP determinations from genomic DNA from 132 patients. They also used the Luminex xMAP™ system and concluded that the xTAG™ platform is a highly accurate, multiplexed, high-throughput SNP-detection assay. Although premature, an extended panel of high, moderate and low-risk alleles could eventually be developed into a useful melanoma risk profiling tool.

There are several limitations to our analysis. Like other allele-specific platforms, variants are interrogated by the bead array rather than discovered. Rare missense mutations, in-frame

insertions, large deletions and mutations not included in the assay cannot be successfully recovered. For example, the common 1–8dup8 mutation, in which the first eight amino acids of exon 1α are exactly duplicated, could not be incorporated into the assay as wild-type and mutant sequences cannot be distinguished from each other by oligonucleotide differences. For mutational screening, several studies, including those from GenoMEL, have now reported successes with dHPLC in identifying *CDKN2A* mutations(Edmunds, et al., 2002; Harland, et al., 2008; Lang, et al., 2005; Marian, et al., 2005; Orlow, et al., 2001). Although direct sequencing and other conformation-based screening strategies, such as dHPLC and high resolution thermal melt (HRM) analysis, unquestionably drive discovery of novel mutations, they are limited in their multiplexing capabilities. Lastly, the maximal utility of this bead array depends on the distribution and frequency of *CDKN2A* and *CDK4* mutations. The GenoMEL consortium enriches for familial cases that have a reasonable founder composition. In some ways, this is an ideal collection to initially validate the assay since a limited panel of variants may in fact capture a significant fraction of cases. However, broader application to the general CMM population may not be appropriate given the current iteration of the assay. In the future, it is possible that other major predisposition genes will be discovered and cost-effective, rapid, high-throughput methods of analyzing populations for founder changes will be developed. The current system reported here can in fact meet this challenge as more genetic information is uncovered.

In conclusion, we have developed and validated the use of a multiplex bead-based assay for the detection of 39 variants associated with familial CMM in the GenoMEL consortium. This multiplex bead-based assay is a fast, sensitive, high sample throughput, cost-effective, and a reliable method for the screening of *CDKN2A* and *CDK4* mutations in a large number of samples. This assay can be easily expanded to include other variants as they are reported and can be readily adapted to specific mutations in specific populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

CMM	cutaneous malignant melanoma
CDKN2A	cyclin-dependent kinase inhibitor 2A
CDK4	cyclin-dependent kinase 4
ARF	alternative reading frame
MC1R	melanocortin 1 receptor
dHPLC	denaturing high-performance liquid chromatography
SNPs	single-nucleotide polymorphisms

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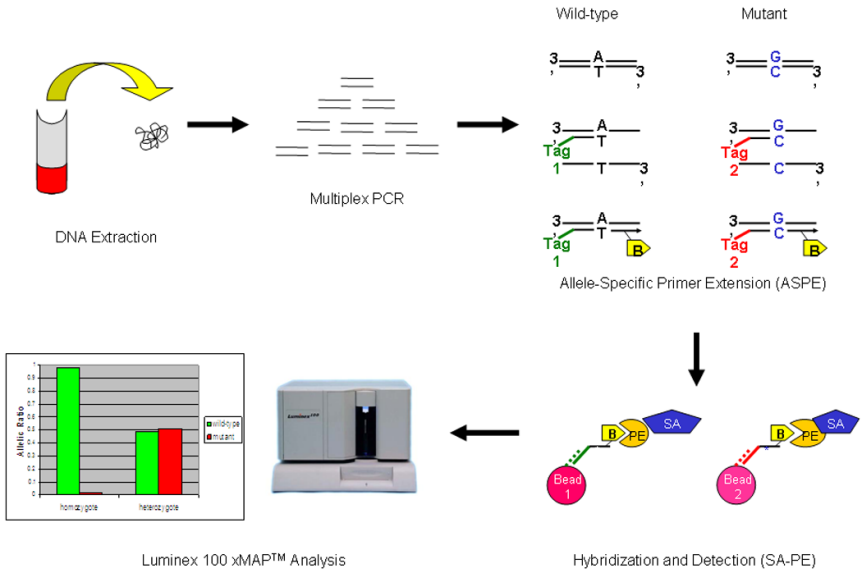

Figure 1. The Bead-based Assay

Regions of interest are initially amplified by multiplex PCR (for *CDKN2A*, the primers are listed in Table S1). Individual probes with the interrogative terminal codon are linked to a unique 24-mer Tag and subjected to ASPE. The ASPE extension step uses biotinylated dCTPs thereby introducing a biotin-labeled moiety into the extended fragment. The 24-mer Tag/biotinylated ASPE fragment is then specifically captured by a color-coded bead adorned with an anti-Tag which fully complements the 24-mer Tag sequence. Streptavidin-conjugated phycoerythrin (SA-PE) is then introduced to detect an intensity signal. In summary, the bead color defines the address (i.e. the individual SNP) while the PE fluorescence intensity defines the amount of the specific SNP fragment.

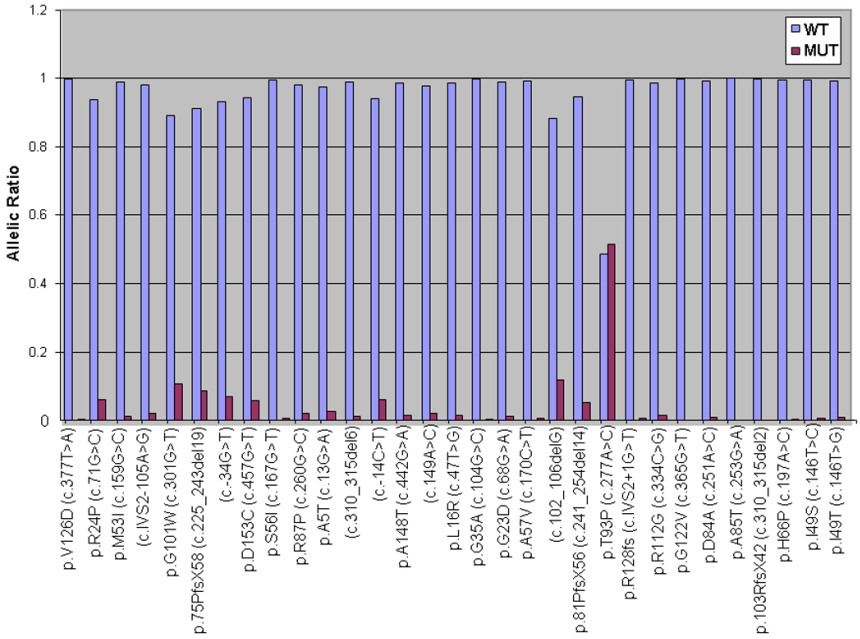

Figure 2. Example of a heterozygous p.T93P mutation in an individual
The allelic ratio is approximately 0.5, as expected in a heterozygous state.

TABLE 1

	Total	Failed reaction	Samples scored	Total variants	1–8dup8 mutations (not in assay)	Unprobed variants	Probed variants	Correct variants scored	Missed variants	Total SNPs interrogated	SNPs assigned correctly
Toronto	1040	22	1005	158	13	0	145	145	0	39195	39195
Barcelona	46	9	37	25	2	7[a]	16	16	0	1443	1443
NCI-US	62	1	61	43	3	4[b]	36	33	3[h]	2379	2376
NCI-Italy	12	1	11	5	0	2[c]	3	3	0	429	429
Genoa	14	2	12	13	0	2[d]	11	11	0	468	468
Leiden	25	1	24	10	0	2[e]	8	8	0	936	936
Queensland	150	10	140	35	3	8[f]	24	24	0	5460	5460
Leeds	226	6	220	57	7	0	50	50	0	8580	8580
Boston	93	0	93	12	2	3[g]	7	5	2[i]	3627	3625
Validation	628	30	598	200	17	28	155	150	5	23322	23317
								0.9677			0.9997
Total	***1668***	***52***	***1603***	***358***	***30***	***28***	***300***	***295***	***5***	***62517***	***62512***
								0.9833			**0.9999**

Affected variants:

[a] p.D84Y, p.L65P, p.R87W (4), p.V59G

[b] p.N71S (2), p.R58X (2)

[c] p.L65P (2)

[d] p.R58X (2)

[e] p.G23R (2)

[f] p.L16P (2), p.L32P (2), p.Q50R (2), p.V51F (2)

[g] p.W15X (2), p.A60T

[h] c.241_254del14 (2), c.IVS2+1G>T

[i] p.A148T(2)