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Tandem *BRAF* Mutations in Primary Invasive Melanomas

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The *RAS/RAF/MAPK* pathway likely mediates critical cell proliferation and survival signals in melanoma. *BRAF* mutations have been found in a high percentage of melanoma cell lines and metastases; however, only a few studies with a limited number of specimens have focused on primary melanomas. We examined *BRAF* exon 15 mutational status in 37 primary invasive melanomas of varying thicknesses, which had undergone a standardized pathology review. *BRAF* mutational status was determined using direct manual sequencing of PCR products, followed by resequencing separately amplified DNA aliquots to confirm each mutation. *BRAF* exon 15 mutations were found in 17 of 37 (46%) primary melanomas. Tumor-specific tandem mutations, encoding either V599K, V599R, or V599E, were found in 5 of 17 (29%) melanomas with *BRAF* exon 15 mutations. Cloning of *BRAF* double base-pair substitutions confirmed that both base changes were on the same allele and can result in a positive charge at codon 599. *BRAF* mutations, including tandem mutations, were frequently found in both thin and thick primary melanomas, implying that these mutations can occur early in the progression of melanoma. The finding of tandem mutations in thin melanomas makes it more likely that they arise as a simultaneous rather than sequential event.

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Melanoma, which frequently metastasizes and generally is resistant to medical treatment, more than tripled in incidence among Caucasians between 1980 and 2003, bringing the estimated number of new invasive melanomas to 54,200 with 7600 deaths for the year 2003 in the United States (Jemal *et al*, 2003). Due to the increasing burden of this potentially lethal disease, methods of prevention, early diagnosis, and treatment of melanoma are expected to be of increasing importance. Improved understanding of the molecular alterations present in primary cutaneous melanoma and the point in melanoma progression that they occur should facilitate advances in these areas.

BRAF encodes a serine/threonine kinase downstream of *RAS* in the *RAS/RAF/MAPK* pathway that is involved in transduction of mitogenic signals from membrane receptors to the nucleus. Through a systematic genomewide screen for mutations in signaling pathways where at least one gene was mutated in human cancer, somatic mutations in the *BRAF* gene in melanomas were found (Davies *et al*, 2002).

BRAF mutations have been found in approximately 40%–80% of melanomas and nevi (Brose *et al*, 2002; Davies *et al*, 2002; Dong *et al*, 2003; Gorden *et al*, 2003; Kumar *et al*, 2003; Pollock *et al*, 2003; Uribe *et al*, 2003). One group, however, reported finding *BRAF* exon 15 mutations in only two of ten (10%) of radial growth phase (RGP) melanomas, whereas they found *BRAF* mutations in five of eight (75%) of vertical growth phase (VGP) melanomas (Dong *et al*, 2003). RGP melanomas can either be *in situ* or have single cells or small clusters of tumor cells without mitoses in the papillary dermis; whereas VGP melanomas would show the opposite, such as cellular clusters in the dermis larger than the largest intraepidermal nest and/or mitoses in the dermal melanoma cells. These authors conclude that *BRAF* mutations are involved in progression rather than initiation of melanoma (Dong *et al*, 2003).

The most common *BRAF* mutation, accounting for 92% of *BRAF* mutations in the melanoma samples tested by Davies *et al* (2002), is that of the 1796T → A transversion in exon 15, which results in a V599E substitution. The *BRAF* V599E variant was demonstrated to transform NIH3T3 cells 138 times more efficiently than wild-type (wt) and to have 10.7-fold increased basal kinase activity (Davies *et al*, 2002). Importantly, using RNA interference experiments, it has been shown that *BRAF* V599E, when present, is necessary for melanoma cell viability and transformation (Hingorani *et al*, 2003). Thus, compounds that inhibit activated *BRAF* are likely to be efficacious in the treatment of melanoma.

The *BRAF* 599 site, which is in the *BRAF* activation segment, is near T598 and S601, which require phosphorylation for maximal *BRAF* kinase activation (Zhang and Guan, 2000). As has been demonstrated for other protein kinases, Davies *et al* (2002) have suggested that the introduction of negative charges in the activation segment may mimic activating phosphorylations, yielding activated kinase.

Abbreviations: LMM, lentigo maligna melanoma; NM, nodular melanoma; RGP, radial growth phase; SSM, superficial spreading melanoma; VGP, vertical growth phase; wt, wild-type

We previously reported *BRAF* exon 15 double mutations at codon 599 in three primary invasive melanomas.[1] In addition, *BRAF* 599 double mutations have been reported in two other primary melanomas, a few metastatic melanomas, a melanoma cell line, and a congenital nevus (Davies *et al*, 2002; Pollock *et al*, 2003; Dong *et al*, 2003; Gorden *et al*, 2003; Kumar *et al*, 2003; Uribe *et al*, 2003). Gorden *et al* (2003) also reported a tandem base-pair substitution, GG1402/1403TC, encoding a *BRAF* G468S amino acid change in a metastasis. No detailed clinical-pathologic data has previously been reported for tandem mutations in primary melanomas.

In this study, we examined a series of 19 hospital-based and 18 population-based primary cutaneous invasive human melanomas, including thin ($\leqslant$0.75 mm Breslow depth) and thick melanomas, which had undergone a standardized pathology review. *BRAF* exon 15 mutational status was determined using manual sequencing, followed by resequencing a separately amplified DNA aliquot to confirm the mutation. Furthermore, double *BRAF* mutations that were found were cloned to determine if they occurred together on the same allele or were separate point mutations on different alleles.

Results and Discussion

Our study finds *BRAF* mutations to be frequent in primary melanomas, including both thin and thick melanomas. Of the 37 primary melanomas evaluated, 17 (46%) were found to harbor *BRAF* exon 15 alterations, all resulting in amino acid changes, as shown in Table I. A high prevalence of *BRAF* exon 15 mutations was found in both the hospital-based (37%) and the population-based (56%) series. The melanoma depths differ between these two groups, with the hospital-based samples being of substantially thicker mean Breslow depth than the population-based samples, consistent with The University of North Carolina Hospitals being a tertiary referral center.

Of note, seven of ten (70%) of the thin melanomas ($\leqslant$0.75 mm) had *BRAF* exon 15 mutations. Although two of these had histologically contiguous nevi that may not have been entirely separated out from the melanoma in the dissection process, five had no associated nevi. In those cases, analysis would represent the mutational status of the melanoma alone. Our study cannot be directly compared with that of Dong *et al* (2003), as they included melanoma *in situ* as part of their RGP melanoma group and did not report Breslow depth on their VGP group; however, our study finds that *BRAF* mutation is frequent once melanomas have early invasion ($\leqslant$0.75 mm Breslow depth).

Our data indicates that tandem mutations comprise a substantial percentage (29%) of the *BRAF* exon 15 mutations found in primary melanomas. Tandem mutations were similarly found in both the population-based and the hospital-based series, with five of 17 (29%) of the samples with *BRAF* exon 15 mutations containing tandem mutations. A 1795–96GT $\rightarrow$ AG change resulting in V599R, two 1795–96GT $\rightarrow$ AA alterations resulting in V599K, two 1796–97TG $\rightarrow$ AA changes resulting in V599E, and a novel 1779T $\rightarrow$ A alteration resulting in D593E were discovered. Figure 1 shows sequencing results of *BRAF* exon 15 in two of the primary melanoma samples that displayed double mutations, the positive control cell line, Colo 205, which carries the V599E mutation (Davies *et al*, 2002), and a negative control from peripheral blood.

We confirmed by cloning that these double mutations are in tandem positions on the same allele and, therefore, can result in positively charged amino acid substitutions. One of each of the melanomas carrying V599K, V599R, and V599E tandem mutations was cloned and sequenced and the double base changes in each case were shown to occur on the same allele, rather than being two separate point mutations on different alleles or resulting from a heterogeneous population of cells with different mutations. Automated sequencing analyses of cloned exon 15 PCR fragments from melanomas with a V599K and V599R tandem mutations are shown in Fig 2. The cloning confirms the amino acid sequence of the *BRAF* tandem mutations and verifies that these mutations can result in positively charged substitutions (V599R and V599K) in some samples and negatively charged amino acid substitutions (V599E) in others. This is in agreement with Gorden *et al* (2003) who cloned and sequenced a GT/AG *BRAF* V599R double mutation from a melanoma metastasis and found both base-pair substitutions to be on the same allele.

Previous reports have suggested that oncogenic mutations in *BRAF* introducing negative charges in the activation segment of exon 15 may be mimicking activating phosphorylations (Davies *et al*, 2002). Because the tandem mutations found can result in lysine and arginine substitutions in the activation segment, as confirmed by cloning, our results suggest that mutations creating positive charges in this segment of *BRAF* exon 15 may stabilize active conformations of the kinase, a finding that has mechanistic implications. In support of this, Dong *et al* (2003) found that the *BRAF* V599K mutant has transforming activity in NIH3T3 cells and can produce rapidly growing tumors in SCID mice (Dong *et al*, 2003).

We have shown that the tandem mutations are tumor specific. Areas of normal-appearing skin adjacent to melanomas from the hospital-based series carrying *BRAF* tandem mutations resulting in V599K, V599R, and V599E alterations were also isolated and analyzed for mutations. No mutations were found in areas of normal skin from these samples, consistent with the *BRAF* tandem gene alterations being tumor-specific somatic mutations rather than germline polymorphisms. Germline DNA or normal tissue from cases with *BRAF* melanoma mutations have been found to be negative for the *BRAF* alteration in other studies (Davies *et al*, 2002; Yuen *et al*, 2002; Gorden *et al*, 2003; Uribe *et al*, 2003).

Notably, tandem mutations were found in primary melanomas as thin as 0.38 and 0.40 mm Breslow thickness, Clark level II and in a melanoma that was still in RGP. These findings provide evidence that these tandem mutations occur early in the progression of melanoma, making it more

[1]Thomas NE, Alexander A, Edmiston S, Parrish E, Millikan RC, Groben P, Ollila D, Conway K: Tandem *BRAF* mutations in primary human melanomas. Proc Am Assoc Cancer Res (2nd ed.) 44:1357, 2003 (abstr).

Table I. BRAF exon 15 mutations in primary cutaneous invasive melanomas and their associated clinicopathologic features

Sex	Age (y)	Nucleotide	Amino acid	Histologic subtype	Breslow depth (mm)	Clark level
Hospital-based series						
M	81	GT1795–96AG	V599R	SSM	3.65	IV
M	71	GT1795–96AA	V599K	SSM	3.50	IV
M	47	TG1796–97AA	V599E	UNCL	10.00	V
F	33	T1796A	V599E	SSM	0.82	III
M	38	T1796A	V599E	SSM	5.70	IV
M	31	T1796A	V599E	SSM	0.75	IV
M	68	T1779A	D593E	NM	2.60	IV
F	89	wt	–	SSM	4.60	V
F	76	wt	–	UNCL	1.30	III
F	26	wt	–	NM	1.00	III
F	43	wt	–	SSM	0.59	II[a]
F	35	wt	–	SSM[b]	1.30	IV
F	78	wt	–	SSM	4.60	V
M	82	wt	–	LMM	1.78	IV
M	83	wt	–	LMM	3.65	IV
M	31	wt	–	NM	1.53	IV
F	70	wt	–	SSM	0.93	IV
F	76	wt	–	SSM	1.25	III
M	58	wt	–	LMM	13.65	V
Population-based series						
F	60	GT1795–96AA	V599K	SSM	0.40	II[a]
F	32	TG1796–97AA	V599E	SSM[c]	0.38	II
F	32	T1796A	V599E	SSM	0.85	IV
M	31	T1796A	V599E	SSM[d]	1.00	III
M	56	T1796A	V599E	SSM	0.70	III
F	34	T1796A	V599E	SSM	0.52	IV
F	59	T1796A	V599E	SSM	1.20	IV
F	45	T1796A	V599E	SSM	0.99	IV
M	25	T1796A	V599E	SSM	0.44	III
M	52	T1796A	V599E	SSM	0.58	III
F	74	wt	–	SSM	1.60	IV
F	51	wt	–	SSM	2.10	IV
F	68	wt	–	NM	3.40	IV
F	78	wt	–	NM	2.50	IV
M	58	wt	–	SSM	0.70	IV
F	27	wt	–	SSM	2.20	IV
F	38	wt	–	SSM	0.55	II
F	73	wt	–	SSM	0.88	IV

wt, wild-type *BRAF* allele detected; SSM, superficial spreading melanoma; UNCL, unclassified melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma.

[a]These two melanomas were in RGP = radial growth phase; all others had a VGP = vertical growth phase.

[b]Histologically contiguous with a dysplastic nevus.

[c]Histologically contiguous with a common acquired nevus.

[d]Histologically contiguous with a congenital nevus.

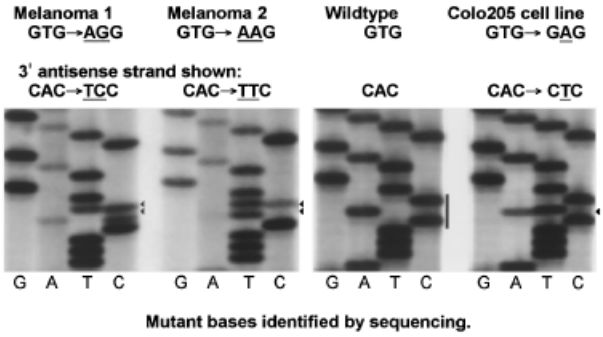

Figure 1
Sequencing analysis of *BRAF* codon 599 mutations in primary melanomas. Melanoma 1 exhibits a double mutation in codon 599 of GTG to AGG, whereas melanoma 2 exhibits a different double mutation in codon 599 of GTG to AAG. The negative control is normal DNA from peripheral blood. The positive control Colo205 displays a single base change of GTG to GAG in codon 599. Arrows indicate mutant bases identified by sequencing.

likely that they occur as a simultaneous rather than sequential event.

In the literature, tandem base mutations that are not opposite bi-pyrimidines appear to be rare in human tumors; a literature search did not reveal any such reported mutations other than *BRAF* tandem mutations. Besides being found in melanoma, tandem *BRAF* V599D mutations have also been reported in two of 15 cholangiocarcinomas with *BRAF* exon 15 mutations, but none have been reported in other tumor types (Tannapfel *et al*, 2003).

Lee *et al* (2002) suggest that tandem mutations could occur through several possible mechanisms, including: (i) a single-base lesion promoting a double misincorporation event during DNA replication; (ii) cross-linked adjacent DNA bases; or (iii) adjacent base lesions (tandem lesions) promoting misincorporation opposite to the two lesions. In addition, tandem mutations have been associated with deficient nucleotide excision repair (Lee *et al*, 2002). It is possible that tandem mutations may arise due to a deficiency in nucleotide excision repair (Lee *et al*, 2002) or a lack of fidelity in this process. The majority of the *BRAF* mutations reported are at codon 599 (GTG) of the sequence 5′-GTG AAA-3′. The adjacent di-pyrimidines on the non-coding strand may form dimers in response to ultraviolet damage and mutations at the codon 599 may occur during DNA repair. Mutations near but not directly opposite bi-pyrimidines have been found in response to ultraviolet light in *Escherichia coli* (Oller *et al*, 1992).

Additionally in our series, a novel *BRAF* D593E mutation was found in a nodular melanoma (NM) with 2.6 mm Breslow thickness. The wt aspartic acid residue at this position is in the DFG (AspPheGly) sequence at the start of the activation segment and is highly conserved in all protein kinases. This aspartic acid residue is involved in binding an ATP chelating metal and has been identified as the catalytic residue in phosphorylase kinase due to its proximity to

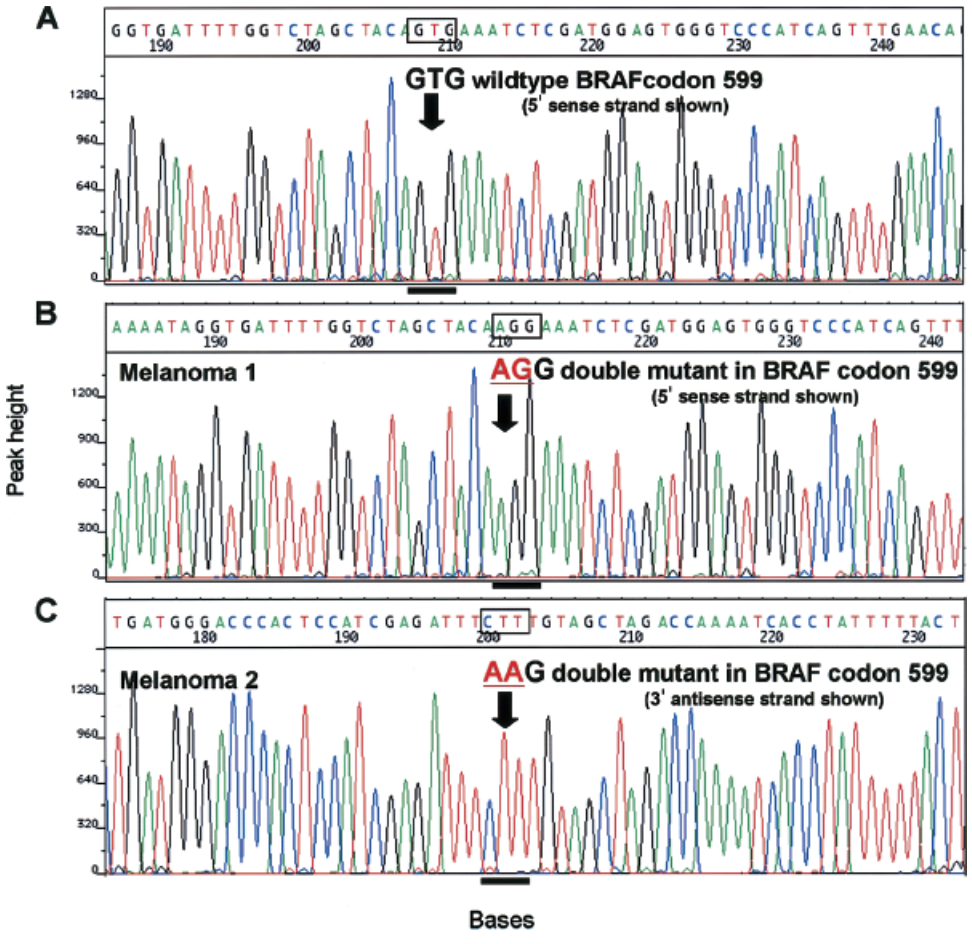

Figure 2
Cloning and automated sequence analysis of *BRAF* codon 599 double mutants from primary melanomas. After cloning the PCR fragments of mutated *BRAF*, the altered bases in the melanomas with double mutations were found to be on the same DNA strand. (*A*) Wild-type GTG sequence at codon 599 is shown. (*B*) Melanoma 1 can be seen to have a GTG to AGG alteration at codon 599. (*C*) Melanoma 2 can be seen to have a GTG to AAG alterations at codon 599.

substrate in the catalytic site (Johnson *et al*, 1998). Substitution of alanine for aspartic acid at position 593 in *BRAF* eliminates kinase activity (Davies *et al*, 2002). *BRAF* D593V and D593G substitutions have been found in colorectal cancers (Yuen *et al*, 2002; Wang *et al*, 2003).

BRAF 599 mutations in melanomas were analyzed for potential associations with clinicopathologic features. The *BRAF* D593E mutant was not included in the analysis as mutations at position 593 can eliminate kinase activity and, thus, it is unclear whether or not *BRAF* D593E is an activating mutation. Our data show a statistically significant association of *BRAF* 599 mutation in primary invasive melanomas with superficial spreading melanoma (SSM) ($p = 0.03$) *versus* other histologic subtypes. The frequency of *BRAF* 599 mutation was found to be significantly greater in SSM melanomas (15 of 27; 56%) than in melanomas of other subtypes (one of eight; 13%). Specifically, in our series, all melanomas with *BRAF* 599 mutations were SSM, with the exception of one unclassifiable melanoma. No statistically significant correlations were detected between *BRAF* 599 mutational status and age, anatomic site, Clark level, solar elastosis, and nevus association in these melanomas (data not shown). There was a borderline negative association between *BRAF* mutational status and increasing Breslow depth ($p = 0.05$). Our data show a possible but not statistically significant association of *BRAF* mutation in primary melanomas with the male gender ($p = 0.09$). This is of note as Uribe *et al* (2003) have found an association of *BRAF* mutational positivity in atypical nevi and melanomas with the male gender. Further examination of a larger population-based series of melanomas will allow a more thorough evaluation of frequency of *BRAF* mutation, including the subset of tandem mutations and potential associations with clinicopathologic variables.

Intermittent, recreational type sun exposure has been linked to most subtypes of melanoma, except for lentigo maligna melanoma (LMM), which is associated with chronic or occupational exposure (Elwood and Jospon, 1997; Armstrong and Kricker, 2001). Both UVB and UVA have been implicated as possible etiologic agents, and oxidative damage has also been suggested as a possible source of DNA damage in melanoma. Whether the type of tandem mutation reported in this study represents a "fingerprint" for an etiologic process remains to be elucidated. Further study of these tandem mutations in population-based melanoma samples along with epidemiologic data may ultimately shed light on the processes involved in the development of melanoma.

Materials and Methods

Sample selection and pathology review The samples consisted of 37 primary invasive cutaneous human melanomas. Nineteen of these were archived primary human melanomas selected at random from a series of cases treated at UNC Hospitals after 1995, which had Breslow thicknesses from 0.59 to 13.65 mm (mean 3.3 mm). An additional 18 melanoma samples came from a larger population-based series in North Carolina in the year 2000, which had Breslow thicknesses from 0.38 to 3.40 mm (mean 1.2 mm). These tumors were selected from this population-based sample set based on sequential date of receipt of the tissue blocks that fulfilled the study criteria of primary cutaneous invasive melanoma in the year 2000 in designated counties of North Carolina and size of tumor dense enough so as not to require laser capture microdissection. Of 24 sequential cases of melanoma, six cases (all $\leqslant 0.6$ mm) were put aside for laser capture microscopy, and the other 18 were included in this study. This population-based study is part of an international melanoma study. Both studies were approved by the Institutional Review Board of the University of North Carolina. Review of the histopathology was performed by one dermatopathologist (P. G.) who was blinded to the mutational status of the cases. An unclassified melanoma is one that could not be classified as SSM, NM, LMM, or acral lentiginous.

Preparation of DNA from melanomas After sectioning of the paraffin-embedded tumor blocks obtained from the cases, the areas of melanoma were identified by a dermatopathologist (P. G.) and dissected from the surrounding tissue under $\times 2$ magnification. Normal appearing skin was also dissected off the slides and isolated separately for the hospital-based samples carrying tandem *BRAF* mutations. Tissues were incubated for 4 h at 56°C in lysis solution containing 1 mg per mL proteinase K, 1% Triton-X, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 0.001% gelatin, then were incubated overnight at 37°C. Proteinase K was then inactivated by heating to 95°C for 10 min. The lysates were centrifuged for 10 min at 13,000 g to remove debris and subjected to molecular assays for *BRAF* exon 15 mutations as described below.

Polymerase chain reaction *BRAF* exon 15 was amplified by PCR in a 50 μL reaction volume containing 1–5 μL of tumor DNA lysate, 400 nmol of each primer (forward: 5′-TCATAATGCTTGCTCTGATAGGA, primer 1; reverse: 5′-GGCCAAAAATTTAATCAGTGGA, primer 2) (Davies *et al*, 2002), 1.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California), 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.001% gelatin), and 125 μM each dNTP. PCR was carried out for one cycle of 95°C for 4 min., followed by 35 cycles of 95°C for 30 s, 57°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 10 min for one cycle. The quantity of each PCR product obtained was analyzed on 10% polyacrylamide gels.

Sequencing The PCR products were purified by electrophoresis in 10% polyacrylamide gel. Bands were isolated from the gels and incubated overnight in 500 μL TE, then were purified using a Promega Wizard PCR purification system (Promega, Madison, Wisconsin). Two asymmetric PCR reactions were prepared to generate two single-stranded DNA products in both the forward and reverse directions. The first asymmetric PCR reaction consisted of 300 nM of primer 1 and 6 nM of primer 2, whereas the second reaction contains 6 nM primer 1 and 300 nM primer 2. All other reaction conditions were the same as described for first round PCR. The amplified single-stranded product was prepared for sequencing by filtration through Centricon 100 spin filters (Amicon, Houston, Texas). The product was sequenced with the Sequenase 2.0 dideoxy-termination method using ^{35}S-dATP to visualize the bands. All mutations were confirmed by sequencing in a separately amplified aliquot of DNA to rule out artifactual mutations.

Cloning of *BRAF* exon 15 mutants *BRAF* exon 15 was cloned into the pDrive vector (Qiagen, Valencia, California), and plasmid DNA minipreps were prepared from each clone using Qiaprep spin minipreps (Qiagen). DNA was sequenced at the UNC-CH Automated DNA Sequencing Facility on a 3100 Genetic Analyzer (Applied Biosystems).The sequencing reaction was done using the ABI PRISMTM BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Applied Biosystems).

Statistical analysis p-Values were derived from the Fisher's exact test. All statistical analyses were performed using SAS system version 8.02 (Cary, North Carolina).

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