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A Variant in a MicroRNA Complementary Site in the 3′UTR of the *KIT* Oncogene Increases Risk of Acral Melanoma

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Abstract

MicroRNAs are small ~22nt single stranded RNAs that negatively regulate protein expression by binding to partially complementary sequences in the 3′UTRs of target gene mRNAs. Recently, mutations have been identified in both microRNAs and target genes that disrupt regulatory relationships, contribute to oncogenesis and serve as biomarkers for cancer risk. *KIT*, an established oncogene with a multifaceted role in melanogenesis and melanoma pathogenesis, has recently been shown to be up-regulated in some melanomas, and is also a target of the microRNA miR-221. Here we describe a genetic variant in the 3′UTR of the *KIT* oncogene that correlates with a greater than fourfold increased risk of acral melanoma. This *KIT* variant results in a mismatch in the seed region of a miR-221 complementary site and reporter data suggests that this mismatch can result in increased expression of the *KIT* oncogene. Consistent with the hypothesis that this is a functional variant, KIT mRNA and protein levels are both increased in the majority of samples harboring the *KIT* variant. This work identifies a novel genetic marker for increased heritable risk of melanoma.

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Keywords

melanoma; acral; microRNA; SNP; cancer risk; miR-221; KIT

Introduction

Melanoma, a malignancy that arises from melanocytes (most often of the skin), is one of the least common but most fatal forms of skin cancer, representing only 3% of skin cancers but resulting in over 75% of skin cancer deaths. An estimated 69 000 cases of melanoma were diagnosed in the U.S. in 2009 and the incidence of this cancer is increasing (Rigel). Melanoma is treatable if caught at the early, localized stage, with a five-year survival rate of 99%. However, this survival rate drops to just 29% for patients diagnosed with a more advanced stage, such as an ulcerated tumor with lymph node involvement, highlighting the importance of early detection and risk assessment (Gershenwald et al.). Approximately 10% of all melanoma cases are thought to be hereditary. For example, family linkage studies identified an autosomal-dominant, melanoma gene located on chromosome 9p21.4. This gene, called CDKN2A (also known as p16 or INK4A or MTS1), accounts for up to 40% of these hereditary melanoma cases (Meyle & Guldberg, 2009). However, other genetic mutations accounting for inherited melanoma risk are unknown.

MicroRNAs (miRNAs) are small ~22nt single-stranded RNAs that negatively regulate protein expression by partially complementary binding usually to the 3′ untranslated region (UTR) of mRNAs of target genes. MiRNAs have been demonstrated to have significant roles in a wide range of cellular processes including development, aging, immunity and disease. In particular, miRNAs have been identified as key players in a virtually all cancers studied, acting as both tumor suppressors and oncogenes (Medina & Slack, 2008; Stefani, 2007). Sequence conservation of miRNAs across species is strong, as is conservation of miRNA binding sites in the 3′UTRs of target genes (Lee *et al.*, 2007).

A key regulator of melanocyte development, the *KIT* oncogene, has recently been identified as a target of miR-221 in melanoma (Felicetti et al., 2008; Igoucheva & Alexeev, 2009). KIT is a receptor tyrosine kinase (RTK) that binds the ligand Stem-Cell Factor (SCF), also known as mast cell growth factor and steel factor (SF) (Smalley *et al.*, 2009b). KIT activation drives a number of downstream pathways associated with malignant transformation, including the PI3K/AKT pathway, known to be important for melanoma progression, as well as the JAK/STAT and MAPK pathways (Smalley *et al.*, 2009b). It is therefore not surprising that aberrant KIT expression and signaling has been described in multiple cancers (Went *et al.*, 2004). For example, KIT expression is particularly robust in gastrointestinal stromal tumors (GISTs), for which immmunohistochemical detection of KIT positivity is considered a prerequisite for diagnosis, and treatment of this type of cancer has seen significant success with the KIT-inhibitor Imatinib (Demetri *et al.*, 2002). However, the role of KIT in melanoma appears complex. KIT, which in some studies seems a promising candidate oncogene in melanoma, has in earlier work been shown to have decreased expression in large number of cutaneous melanomas relative to primary melanocytes (Funasaka et al., 1992; Lassam & Bickford, 1992; Natali et al., 1992; Went et al., 2004), and

in some studies either exogenous re-expression of the receptor, or addition of the ligand to the culture medium of KIT-expressing melanoma cells, can inhibit growth in cell culture (Huang et al., 1998; Huang et al., 1996; Zakut et al., 1993). Conversely, recent studies have shown positive KIT expression in 36% of melanomas examined (Went *et al.*, 2004), and have demonstrated KIT signaling as the driving oncogenic event in some sub-groups of melanoma, which harbor activating mutations or amplifications of *KIT* (Curtin et al., 2006; Smalley et al., 2009a; Smalley et al., 2009b). In particular, KIT expression appears to be associated with acral melanoma (Ashida *et al.*, 2009; Curtin *et al.*, 2006), which occurs on the distal parts of the body and mucosal surfaces. Activating mutations or amplifications of *KIT* have been found in 39% of mucosal and 36% of acral melanomas (Curtin *et al.*, 2006). While previous trials using Imatinib to treat melanoma were negative, a body of recent work suggests that this drug and similar RTK inhibitors may have promise with respect to this class of KIT-driven cancers (Ashida *et al.*, 2009; Jiang *et al.*, 2008).

Recent work from several laboratories has examined the effects of mutations in miRNAs and their targets, and have identified genetic markers of cancer risk (Mishra & Bertino, 2009). For example, a single-nucleotide polymorphism (SNP) in the miR-146a precursor results in production of polymorphic pre-miRNAs from the passenger strand and predisposes to papillary thyroid carcinoma (Jazdzewski *et al.*, 2009). A SNP in the miR-125b binding site in the *BMPR1B* 3′UTR disrupts miRNA repression of this target and confers increased risk of breast cancer (Saetrom *et al.*, 2009). We found that a SNP in a *let-7* miRNA complementary site in the 3′UTR of the *KRAS* oncogene disrupted base-pairing in the miRNA:target duplex and significantly increased risk of non-small cell lung cancer (NSCLC) (Chin et al., 2008) and ovarian cancer (Ratner et al., 2010). Given these examples, we speculated that 3′UTR variants might play roles in the development and pathogenesis of melanoma.

Here we show that a genetic variation in a miR-221 complementary site in the *KIT* 3′UTR correlates with increased risk of melanoma, specifically of the acral subtype, and propose this variant allele as a new genetic marker for acral melanoma risk.

Results

A variant allele in a miR-221 complementary site in the *KIT* 3′UTR

We amplified the 3′UTR of the KIT oncogene from genomic DNA of 70 melanoma patients, and examined this region for sequence variability. As all but one of available melanoma samples were taken from patients of European descent, the study was limited to this group. We found a high frequency of heterozygosity for a variant/derived “A” allele at *rs17084733* (3169G→A) (24.3%) in our samples relative to 16.7 to 23.3% in various European (“Caucasian”) samples as reported in dbSNP (Sayers *et al.*, 2009; Sherry *et al.*, 2001). The *KIT* 3′UTR has been validated as a target of miR-221 by reporter assays (Felicetti et al., 2008; Igoucheva & Alexeev, 2009), and three binding sites have been proposed based on computational prediction software. The variant allele at *rs17084733* (referred to here as the *KIT* variant) is predicted to disrupt base pairing within the seed region of the 5′-most miR-221 complementary site (He *et al.*, 2005) (Fig. 1A). Interestingly, the *KIT* variant was previously reported in a study examining the correlation between high miR-221 and low

KIT expression in papillary thyroid carcinoma (He *et al.*, 2005). Out of 10 patients with low levels of KIT protein and gene transcript, five were heterozygous at *rs17084733*, an observation that conflicts with the hypothesis that this *KIT* variant would result in increased expression of KIT. Significantly, this miR-221 complementary site is highly conserved in mammals, with the site of *rs17084733* being most conserved, and with the least conserved base lying at a site not predicted to base-pair in the miR-221:target duplex (Fig. 1B).

The frequency of the variant allele in worldwide populations

As reported above, *rs17084733* is a locus with low frequency of heterozygosity in the dbSNP and HapMap databases. To verify that the frequency of the *rs17084733* variant in melanoma exceeded that of the general population and may therefore be a marker of melanoma risk, we examined the genotype of this locus in 2,765 healthy individuals from 58 groups around the world representing African, Southwest Asian, European, Siberian, Central Asian, Pacific Islander, East Asian, North American, and South American populations from a Yale collection (Rajeevan et al. 2005). We found a large range of frequency of the variant allele, spanning from 0% in all South American groups as well as sub-groups from four other populations, to 43.2% in the Papua New Guinea sub-group of the Pacific Islanders (Fig. 2). Range was variable across groups, including Europeans, which had and average frequency of the variant allele of 10.0%, but a range from 1.5% in Finns to 22.9% in Sardinians. European Americans in this panel had an allelic frequency of 10.3%. Our melanoma samples had an allelic frequency of 12.9%, representing a 29.0% and 25.2% increase in frequency of this allele relative the European average and European Americans respectively. However, given the range of frequencies observed, we concluded that a control population closely representative of the population from which the melanoma samples were gathered was required. Note that above, we are reporting allelic frequency rather than frequency of heterozygosity to account for rare homozygotes for this *KIT* variant in large worldwide populations.

Case-control analysis of the *KIT* variant in melanoma demonstrates increased melanoma risk in carriers of the variant allele

As all of our melanoma patients were taken from a Yale/New Haven Hospital patient cohort, we assembled a control for our specific study panel taken from healthy individuals from the same community (Supplementary Table 1). We found a frequency of heterozygosity of the variant allele to be 16.0% (n=94) in this population, whereas the frequency of heterozygosity in melanoma patients is 24.6% (n=69), demonstrating a statistically significant 53.8% increase in the frequency of heterozygosity for this allele in patients with melanoma (p=0.02) (Fig. 3). The odds ratio (OR) for the frequency of heterozygosity in melanoma patients relative to controls, adjusted for gender and age, is 3.30 (95% CI (1.27, 8.86)) indicating that the presence of this *KIT* variant more than triples the risk for melanoma. Taken together these data indicate that this *KIT* variant may be a strong predictive marker for increased risk of melanoma.

The presence of the variant allele correlates specifically with increased risk of acral melanoma

As recent work has indicated that KIT expression is associated with acral melanomas, we had included several tumors of this subtype in our initial patient pool. To determine whether this variant is particularly associated with acral melanoma we compared the frequency of heterozygosity of the *KIT* variant in acral and non-acral subtypes (Supplementary Table 1). We found the frequency of heterozygosity of the *KIT* variant in non-acral samples to be 22.0% (n=41), demonstrating a 38% increase relative to controls; however this difference is not statistically significant (p=0.070). In contrast, the frequency of heterozygosity in the acral subtype was 28.6% (n=28), demonstrating a 78.8% increase in the frequency of heterozygosity of this *KIT* variant in these cancers relative to controls, with an OR of 4.24 (95% CI (1.25, 14.65) p=0.02), indicating that this *KIT* variant may be a strong marker specifically for risk of acral melanoma (Fig. 3).

KIT mRNA and protein in samples harboring the variant allele

To determine the impact of the variant on KIT expression we examined whether the presence of the variant allele correlated with altered levels of KIT mRNA and protein levels in melanoma. Microarray gene expression analyses revealed significantly higher levels of KIT mRNA in samples heterozygous for the variant allele relative to wild-type samples (Fig. 4). While sample numbers were low for these experiments, the maginitude of the difference in expression levels far overcomes the sample size. Similarly, Western blots analyses showed that 60% of samples heterozygous for the *KIT* variant allele were positive for KIT expression, compared to only 24% of wild-type samples, suggesting that the presence of the *KIT* variant may result in increased expression of KIT protein. However given the binary nature of either positive or negative KIT expression and sample size we present this as a correlation rather than a statistically significant difference (Fig. 5). We also found that the expression of miR-221 was slightly higher in patients carrying the variant allele, (Suppl. Fig. 2, Suppl. Table 2), however this difference was not statistically significant. Additionally, miR-221 expression was not decreased in the KIT positive samples relative to the KIT negative samples in either the wild-type or variant group suggesting that the increase in KIT expression in the variant relative to the wild-type group is not merely due to a decrease in expression of miR-221 (Suppl. Fig. 3).

Reporter assays indicate that the variant allele disrupts *KIT* 3′UTR mediated translational repression

To determine how the variant allele affects *KIT* expression *in vitro*, we designed two constructs in which the 3′UTR of *KIT* was cloned into a Renilla luciferase reporter, with and without the *KIT* variant. These constructs were transfected into the 501 mel melanoma cell line, which expresses miR-221 (1.7-fold increase in expression relative to average of melanoma samples tested, (Suppl. Table 2)), and assayed for luciferase activity. The studies demonstrated a 6.7-fold increase in luciferase activity in the presence of the variant allele relative to the wild-type allele (normalized to internal vector Firefly luciferase control, p = 0.0233) (Fig. 6a). To test if this derepression depended on miR-221 and the 5′ miR-221 complemntary site, we knocked-out both the 3′ miR-221 complementary sites in the *KIT*

3′UTR within the above vectors and co-transfected them into the YUSAC2 melanoma cell line along with exogenous miR-221 pre-miR. These studies demonstrated a 1.24-fold increase in luciferase activity in the presence of the variant allele relative to the wildtype allele with downstream miR-221 binding sites knocked out (normalized to scrambled pre-miR control and internal Firefly luciferase control, $p = 0.0389$) (Fig. 6b). Thus, the variant allele causes derepression of the *KIT* 3′UTR *in vitro* and may allow increased KIT expression *in vivo*.

The variant allele is not linked to known KIT activating mutations

KIT activating mutations have been identified in exons 11, 13 and 17 of the coding region. To determine if the variant at *rs17084733* might be merely a bystander linked to one of these mutations, these exons were sequenced in 10 samples carrying the variant allele. No mutations were found.

The variant allele is a germline mutation

As *rs17084733* has been previously reported as a human SNP, it strongly suggests that this SNP is a germline variant, and not a somatic mutation. To test this hypothesis we obtained paired normal lymphocytes for 11 of our melanoma samples carrying *rs17084733.* Of these 10 (91%), also carried this *KIT* variant, strongly supporting the hypothesis that this is overwhelmingly a germline variant, and thus a potential genetic marker for melanoma risk.

Discussion

Here we demonstrate that a variant in the seed region of a miR-221 complementary site in the 3′UTR of the *KIT* mRNA correlates with a greater than four-fold increase in risk for the development of acral melanoma. Significantly, the acral sub-type has recently been shown to be associated with high levels of *KIT* expression. We additionally show that the presence of this *KIT* variant may lead to increased expression of KIT as evidenced via luciferase reporter constructs, which is likely due to decreased base pairing in the seed region of the miRNA:target duplex. Furthermore, this polymorphism is associated with increased KIT mRNA and protein levels in the majority of samples that harbor it. This work therefore identifies this *KIT* variant as a novel genetic marker for risk of acral melanoma, and suggests a mechanism through KIT mis-regulation.

Recent work by several groups presents a convincing argument for a significant oncogenic role for the *KIT* oncogene in at least some sub-classes of melanoma, renewing interest in KIT as a possible therapeutic target in these types, and highlighting the potential of *KIT* mutations to serve as biomarkers for melanoma risk. Multiple studies have found increased KIT expression in acral tumors lacking *KIT* amplifications or mutations (Curtin *et al.*, 2006; Smalley *et al.*, 2008), suggesting other forms of KIT mis-regulation. KIT is critical for the survival, proliferation and migration of melanocytes and precursor cells, and a mouse model of forced expression of constitutively active KIT in melanocytes exhibited increased migration of these cells (Alexeev & Yoon, 2006). These properties combined with its known oncogenic capabilities in other tissues highlight *KIT* as a candidate for a role in the development and pathogenesis of melanoma. However, work showing that KIT expression

declined rather than increased in melanomas relative to primary melanocytes (Funasaka et al., 1992; Lassam & Bickford, 1992; Natali et al., 1992), and that exogenous expression of *KIT* in some KIT-negative melanomas, or the addition of the KIT ligand to KIT positive melanoma cells, could induce apoptosis (Huang et al., 1998; Huang et al., 1996; Zakut et al., 1993), runs contrary to this hypothesis. One study further showed that *KIT* is a target of the miRNA miR-221 and that expression of this miRNA increases over the progression of melanoma subsequent to silencing of its repressor PZLF, leading to down-regulation of KIT expression (Felicetti et al., 2008; Igoucheva & Alexeev, 2009).

However, given the robust oncogenic role of *KIT* in other cancers, combined with the genetic and histological complexity and diversity of melanoma as a cancer class, it is possible that *KIT* plays a transforming or pro-proliferative role in particular subtypes of melanoma, or at particular points in the transformation process, progression, migration or metastasis of the disease. Ours and other recent data support this hypothesis. *KIT* positivity has been shown in some sub-classes of melanoma, in the presence and absence of amplifications and activating mutations of *KIT*, particularly in the acral and mucosal subtypes (Curtin et al., 2006; Smalley et al., 2009b). In one report, *KIT* expression was found in melanomas that lack the activating V600E *BRAF* mutation (Curtin *et al.*, 2006), the most common oncogenic mutation identified in melanomas, found in 42% of tumors (see COSMIC Database, Wellcome Sanger Trust), although this is not the case in our cohort of melanoma where KIT expression did not correlate with BRAF mutation status (Halaban, unpublished). However, of the 11 samples in our study harboring the *KIT* variant that were typed, only two (18%) carried BRAF mutations. Interestingly, one study described a group of KIT and cyclin-dependant kinase 4 (CDK4) positive melanomas with high levels of activated phospho-KIT that did not carry *KIT* mutations, and showed no evidence of a SCF/KIT autocrine loop, and yet exhibit high levels of activated phospho-KIT (Smalley *et al.*, 2008). The authors suggest that signaling activity may have arisen from high *KIT* expression levels leading to spontaneous dimerization and activation of this receptor. Similarily, when we examined KIT phosphorylation status in wild-type samples and samples harboring the *KIT* variant, samples harboring the variant allele were phosphorylated more often and to a greater degree than wild-type samples ($p=0.028$) (Supplementary Fig. 1). We therefore speculate that the high expression of the protein within these cells due to repression inhibition in the presence of the variant allele may be sufficient to promote autoactivation of KIT, however further work is required to investigate this hypothesis.

Another study presented compelling evidence of a requirement for a specific epigenetic environment for *KIT*-dependent transformation, demonstrating that in some melanomas *KIT* cooperates with a hypoxia-inducible factor (HIF-1α) to activate the downstream Ras/Raf/Mek/Erk pathway, and that in the absence of hypoxia or exogenous expression of this factor, *KIT* was unable to induce transformation of melanocytes (Monsel *et al.*, 2009). These authors speculate that this could contribute to the apparent tissue specificity of KIT-driven melanomas, for example if hypoxic conditions exist in the extremities of the hands and feet where KIT-associated acral melanomas can occur. Of particular note, many melanomas expressing high levels of KIT have shown considerable response to the KIT inhibitor Imatinib both in cell-culture and xenograft models (Smalley *et al.*, 2008). Two

patients presenting with metastatic melanoma exhibiting strong KIT expression showed significant response to treatment with Imatinib (Hodi *et al.*, 2008; Lutzky *et al.*, 2008). Phase II trials are currently underway for Imatinib treatment of patients with activating *KIT* mutations (Smalley *et al.*, 2009b).

Our new work identifies a novel genetic marker for risk of acral melanoma, and presents a mechanism via mis-regulation of the *KIT* oncogene. Screening for the presence of this *KIT* variant may aid in early identification of at-risk patients, and may also be useful in predicting patients with enhanced response to treatment with RTK inhibitors, a hypothesis that warrants testing.

Materials and Methods

Sample populations

Tumor and case-control samples were from patients with a diagnosis of melanoma and from healthy individuals, respectively (Supplementary Table), and were collected with patients' signed informed consent according to a protocol approved by the Yale University Human Investigation Committee. Tissue was collected from 70 melanoma patients and 94 controls. To determine the worldwide frequency of the *KIT variant* alleles, 2,765 individuals were genotyped from a global sample of 58 populations. According to population ancestry and geographic locations, these 58 populations are categorized into 9 groups: African, Southwest Asian, European, Siberian, Central Asian, Pacific Islander, East Asian, North American, and South American. Sample descriptions and samples sizes can be found in the Allele Frequency Database (ALFRED) by searching for the population names (Rajeevan *et al.*, 2005). DNA samples were extracted from lymphoblastoid cell lines established and/or grown in the Yale University laboratory of K. K. K. The methods of transformation, cell culture, and DNA purification have been described (Anderson & Gusella, 1984). All volunteers were apparently normal and otherwise healthy adult males or females and samples were collected after receipt of appropriate informed consent.

Sequencing of the *KIT* 3′UTR

DNA was isolated from frozen melanoma tumors from 25 patients using the DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions. The entire 2158 nt *KIT* 3′UTR was amplified using *KOD Hot Start* DNA polymerase (Novagen) according the manufacturer's instructions (cycling conditions: 95 C for 2 minutes; then 35 cycles of: 95 C for 15 seconds, 57.8 C for 15 seconds, 72 C for 1 min 15 seconds; then 72 C for 10 minutes) using 50 nanograms (ng) of DNA, and primers flanking the region (forward primer (SG24): CCATCAGTTAGTTGTGATCTT, reverse primer (SG40) CCAGCTCATACATACTAAGCA). PCR products were purified using the QIAquick PCR Purification kit or 96 PCR Purification kit (Qiagen) according to manufacturer's instructions and sequenced in three overlapping segments using 100 ng of purified DNA per reaction (forward primer segment 1 (SG24): CCATCAGTTAGTTGTGATCTT, reverse primer segment 1 (SG28): GGACATAATGCCAGGGTTGTA, forward primer segment 2 (SG27): GCTCTTCTGTGGACCACTGCAT, reverse primer segment 2 (SG38): GCATAGAACTCCAGTGCAA, forward primer segment 3 (SG39):

GCAAATGTGTACATGGCAGAGTT, and reverse primer segment 3 (SG40): CCAGCTCATACATACTAAGCA). Sequencing was carried out by the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University and sequences returned were viewed and analyzed using DNASTAR Lasergene SeqMan Pro DNA sequence analysis software.

SNP genotyping assays

For high-throughput genotyping of the *rs17084733* locus, DNA was isolated from patient tumor and control samples as above, and was analyzed using a Taqman PCR based SNP assay specific for *rs17084733* (Assay #: C__34674348_10 Applied Biosystems, context sequence: AACTGTATATATTCCCAATAGCAAC[A/G]TAGCTTCTACCATGAACAGAAAACA) according to manufacturer's instructions. Reactions were performed in a 25 microliter (μl) volume with 12.5 μl of 2x Taqman Genotyping Master Mix (Applied Biosystems), 1.25 μl of 20x of the SNP assay probe mixture (above) and 25 ng of purified DNA. Initial testing of the assay was performed by genotyping the 25 previously sequenced patient DNA samples as control, resulting in 100% accuracy relative to sequencing results for this locus. Subsequent assays were performed with the following controls: one reaction with water replacing DNA as negative control, one pre-sequenced wild-type sample, and one pre-sequenced sample heterozygous for *rs17084733*. Reactions were run in 96 well plates on the Applied Biosystems 7900HT Fast Real-Time PCR System, cycling conditions: 1 cycle of 50 C for 2 minutes, 95 C for 10 minutes; 40 cycles of 95 C for 15 seconds, 60 C for 1 minute. Genotypes were analyzed using the Applied Biosystems SDS genotyping software.

KIT 3′UTR reporter construct

A psiCHECK-2 (Promega) derivative containing the entire *KIT* 3′UTR (*KIT* wild-type) was generated as follows. The *KIT* 3′UTR was amplified from human genomic DNA isolated as above using primers that included an *Xho*I and a *Not*I restriction site at the 3′ and 5′ ends of the UTR respectively (3′ end primer (SG43b): AAAAAAAActcgagGCAGAATCAGTGTTTGGGTCA, 5′ end primer (SG44b): AAAAAAAAgcggccgcTTGATTTATATATGTACATTTTATTAG). This fragment was cloned into a TOPO vector using the TOPO TA Cloning Kit (Promega) as according to manufacturers instructions and then sub-cloned into the psiCHECK-2 vector using the *Xho*I and a *Not*I sites in the multiple cloning site downstream from the Renilla luciferase coding region. The mutant variant was generated in the TOPO clone using the site-directed mutagenesis system GeneTailor (Invitrogen) as according to manufacturer's instructions using mutagenic primers specific for *rs17084733* (forward mutagenesis primer (SG49b): ATTCCCAATAGCAACaTAGCTTCTACCAT, reverse mutagenesis primer (SG50b): GTTGCTATTGGGAATATATACAGTTGGAA) and subsequently sub-cloned into the psiCHECK-2 vector as above. Vectors were sequenced as above, using 600 ng DNA. Vectors with downstream miR-221 binding sites knocked out were generated in the TOPO clone using the site-directed mutagenesis system GeneTailor (Invitrogen) as according to manufacturer's instructions using mutagenic primers to replace the seed with adenosine repeats (site 1: forward mutatgenesis primer (SG100): TTGGATTCTTAAAAAAAAGGAAATAAAGTATAGG, reverse mutagenesis primer

(SG105): AAGAATCCAAACTAAGATGGCAGTGTTTTCCCACTCC; site 2: forward mutatgenesis primer (SG102): GTAAATATTGAAAAAAAAAAATAATGTC, reverse mutatgenesis primer (SG103): TTCAATATTTACAAAAAAAGCCAC) and subsequently sub-cloned into the psiCHECK-2 vector as above. Vectors were sequenced as above, using 600 ng DNA.

Cell culture and transfection

501 mel and YUSAC2 melanoma cells were cultured in OptiMEM (Gibco) supplemented with 5% fetal bovine serum and penicillin/streptomycin (Invitrogen). For reporter expression assays, the cells were seeded in 6-well plates at 10^5 cells per well, grown for 48 hours and transfected using DMRIEC transfection reagent (Invitrogen) according to manufacturer's instructions. Cells were incubated with DMRIEC transfection reagent (6.25 μl) and plasmid DNA (250 ng) encoding either wild-type or mutant KIT reporter constructs in OptiMEM (Invitrogen, Carlsbad, CA) in a final volume of 0.5 milliliters (ml) per well for 5 hours. OptiMEM (0.5 ml) supplemented with 10% fetal bovine serum was then added to each well, cells were incubated for 48 hours and collected for luciferase assay. Reporter expression was measured using the Dual-Luciferase Reporter Assay (Promega) according to manufacturer's instructions.

Western blotting

Normal melanocytes and melanoma cells were lysed in buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with a mixture of protease (Complete Boehringer Mannheim Corp., Roche Molecular Biochemicals, Indianapolis, IN) and phosphatase (100 mM NaF, 10 mM $Na_4P_2O_7$, and 1 mM Na_3VO_4) inhibitors. Total cell extracts (8 μg protein/lane), measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), were fractionated in precast gels composed of 4–12% gradient polyacrylamide (NuPAGE Bis-Tris, Invitrogen, Carlsbad, CA) and Western blotted according to standard protocols using rabbit anti-c-KIT antibody (C-19 sc-168, Santa Cruz Biotechnologies, Inc, Santa Cruz, CA).

Microarrays

NimbleGen human whole genome expression microarrays (array 2005-04-20_Human_60mer and array 2006-08-03_HG18_60mer) were probed to determine gene expression in melanoma cells isolated from different tumors as previously described (Halaban *et al.*, 2009; Koga *et al.*, 2009).

miRNA expression analysis

MiRNA levels were determined by low density TaqMan arrays (Applied Biosystems) according to the manufacturer's instructions as described previously (Godshalk et al., 2008).

Statistical analysis

KIT variant frequency data were included in univariate logistic regression models to assess significance. Multivariate logistic regression models were also investigated and included the SNP frequencies plus gender, age and race. Significance of microarray data was assessed via

Student's t-test. Significance of the phosphorylation data was assessed via the Wilcoxon rank sum test.

KIT phosphorylation

Lysates of melanoma cell strains were analyzed using the Proteome Profiler human phospho-RTK arrays (catalogue number ARY001 R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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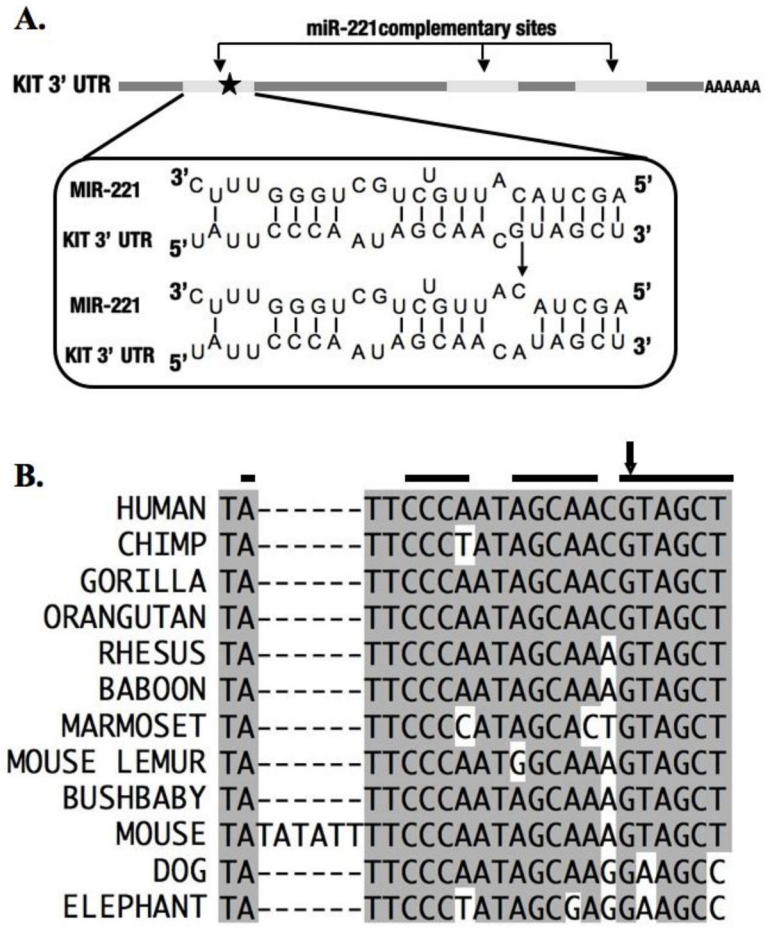

Figure 1. A genetic variant in a miR-221 binding site in the 3′UTR of *KIT*

A. Schematic of the *KIT* 3′ UTR: miR-221 complementary sites are indicated, variant *rs17084733* designated by a star. Inset diagram of miR-221 binding at a complementary site in the wild-type *KIT* 3′ UTR on top, and with *rs17084733* variant allele present on bottom. Wild-type common allele G (top), with variant allele A (bottom) exhibiting decreased basepairing in the seed region of the miRNA::target duplex. In both cases the miR-221 is the bottom strand of the duplex. B. The miR-221 complementary site is highly conserved in mammals. Data extracted from the UCSB Genome Browser. Regions of conservation are shaded, black bar across top indicates regions base-pairing with miR-221, arrow indicates *rs17084733* locus.

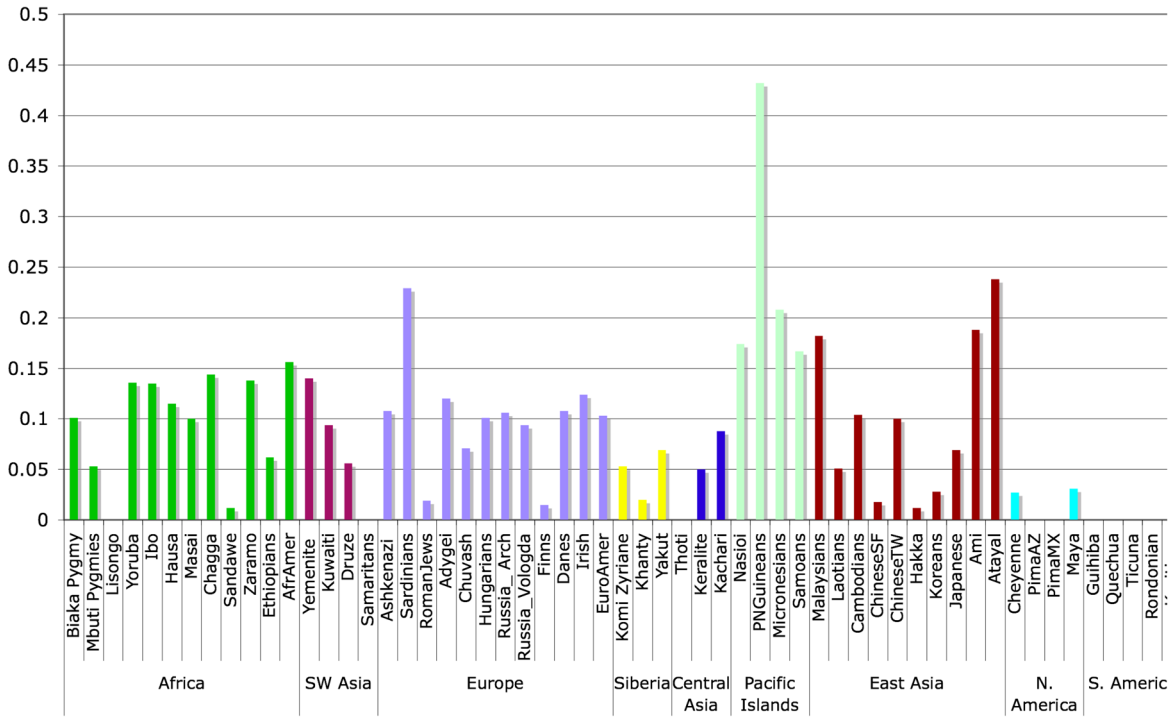

Figure 2. Allelic frequency of the *rs17084733* variant allele in world populations

Frequency of the variant (A) allele at *rs17084733* in 2,765 individuals representing 58 groups from 9 populations around the world.

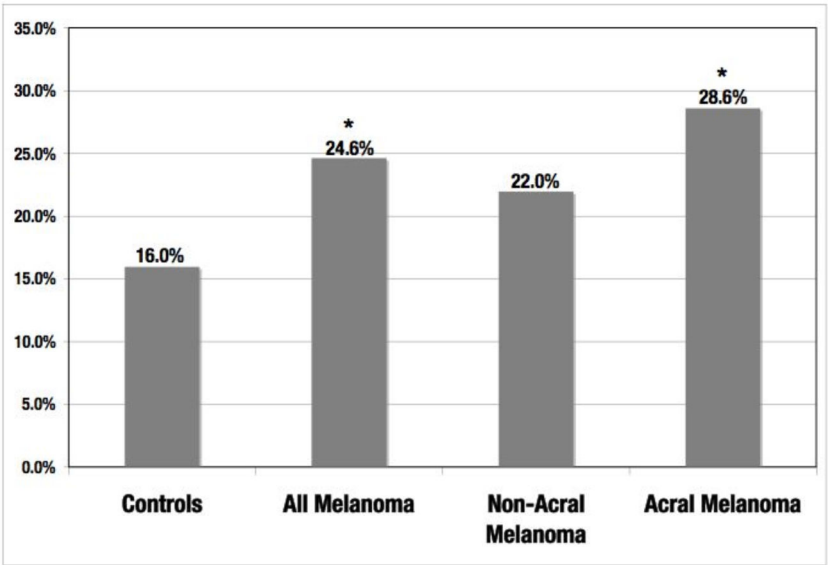

Figure 3. Frequency of heterozygosity of the *KIT* variant in case control, melanoma, and melanoma subtypes

Frequencies of heterozygosity of the variant allele are shown for the Yale-New Haven Hospital patient case-control cohort: all melanoma samples, and divided into non-acral and acral groups. Samples exhibiting a statistically significant difference ($p<0.05$) relative to control group are indicated with an asterix. (p-values: all melanoma=0.02, non-acral=0.070, acral=0.02.)

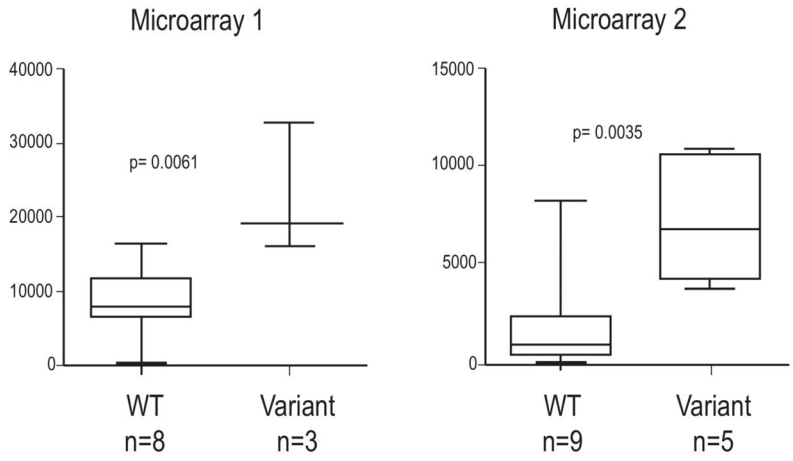

Figure 4. Expression of *KIT* as analyzed in two independent microarrays

Relative mRNA levels in wild-type melanomas and melanomas harboring the *KIT* variant analyzed in two microarray experiments indicate the presence of the *KIT* variant correlates with significantly increased levels of KIT mRNA.

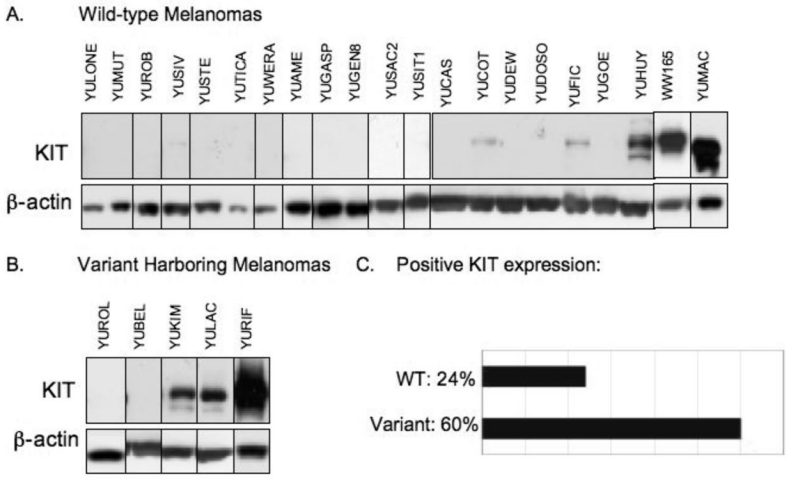

Figure 5. KIT expression at the protein level

Western blot analysis of wild-type (A) and melanomas heterozygous for the *KIT* variant allele (B). (C) A histogram showing that the percentage of samples with KIT positivity in melanomas harboring the variant is twice that of melanoma cells wild-type at this locus.

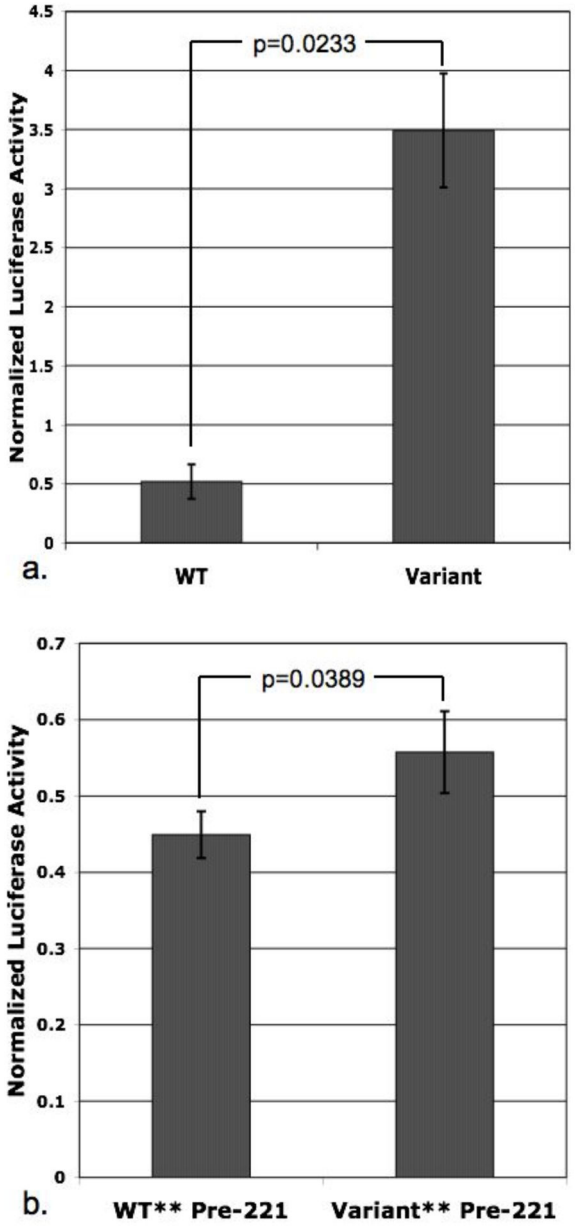

Figure 6. Reporter assays demonstrate an increase in expression from a construct containing the *KIT* variant relative to a wild-type construct

A. Relative luciferase activity normalized to internal vector controls in 501 mel cells transfected with wild-type and variant containing *KIT* 3′UTR reporter constructs indicate a statistically significant 6.7-fold increase in reporter expression from the construct containing the *KIT* variant, providing evidence that this *KIT* variant may result in increased expression of KIT. B. Relative luciferase activity normalized to scrambled pre-miR control and internal vector controls in YUSAC2 cells cotransfected with wild-type and variant containing *KIT* 3′UTR reporter constructs and miR-221 pre-miR also indicate a statistically significant 1.2-fold increase in luciferase activity from the construct containing the *KIT* variant.