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Melanoma cases demonstrate increased carrier frequency of Phenylketonuria/hyperphenylalanemia mutations

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Summary

Identifying novel melanoma genetic risk factors informs screening and prevention efforts. Mutations in the phenylalanine hydroxylase gene (the causative gene in phenylketonuria) lead to reduced pigmentation in untreated phenylketonuria patients, and reduced pigmentation is associated with greater melanoma risk. Therefore, we sought to characterize the relationship between phenylketonuria carrier status and melanoma risk. Using National Newborn Screening Reports, we determined the United States phenylketonuria/hyperphenylalanemia carrier frequency in Caucasians to be 1.76%. We examined three publically available melanoma datasets for germline mutations in the phenylalanine hydroxylase gene associated with classic phenylketonuria and/or hyperphenylalanemia. Mutations were identified in 29/814 melanoma patients, with a carrier frequency of 3.56%. There was a two-fold enrichment ($p\text{-value}=3.4 \times 10^{-5}$) compared to the Caucasian frequency of hyperphenylalanemia/phenylketonuria carriers. These data demonstrate a novel association between phenylalanine hydroxylase carrier status and melanoma risk. Further functional investigation is warranted to determine the link between phenylalanine hydroxylase mutations and melanomagenesis.

Keywords

Phenylketonuria; melanoma; genetics; whole exome sequencing; hyperphenylalanemia

Phenylketonuria (PKU) is a recessive inborn error of metabolism caused by mutations in the phenylalanine hydroxylase (*PAH*) gene. PKU is found more commonly in Caucasian populations, with an approximate incidence of 1:10,000, and, therefore, a carrier frequency

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of around 2% in Caucasians.(Hardelid et al., 2008) Previous studies have shown a lower incidence of PKU in populations with darker skin, particularly of African and South Asian descent. (Hardelid et al., 2008)Furthermore, it is well known that patients with untreated PKU have light hair, eyes and skin.

Functional studies of *PAH* in carriers of pathogenic mutations demonstrate approximately 30% function of the *PAH* enzyme as compared to a control population.(Grimm et al., 1977) While residual *PAH* function is likely responsible for the lack of neurologic phenotype in PKU carriers, it may be functionally significant in other tissues. In melanogenesis, the *PAH* enzyme catalyzes the reaction that yields L-tyrosine, which is the primary precursor for the final melanin products. Also, it has been shown that an increase in phenylalanine concentration as found in PKU patients can have an inhibitory effect on melanin production. (Farishian and Whittaker, 1980) Lastly, elevated phenylalanine levels can increase mitogen-activated protein kinase pathway activation, a critical component within melanoma.(Ayush et al., 2016) Therefore, we hypothesize that mutations in *PAH* may influence susceptibility to melanoma. In this study, we set out to examine whether PKU carrier frequency is enriched in melanoma patients as compared to the expected rates in the Caucasian population, using publically available germline whole exome sequencing data of melanoma patients.

Given the range of PKU/hyperphenylalanemia incidence rates that are reported (and the subsequent effect on estimated carrier frequency in the population), we estimated the specific carrier frequency in the Caucasian population in the United States using newborn screening data. We utilized the National Newborn Screening Reports publically available for the years 1996–2000, which provided a breakdown by race as well. Given varying definitions of hyperphenylalanemia – clinically significant, not clinically significant – as well as classic PKU utilized by different state registries, we combined all of these categories for incidence calculation, as previously described.(Berry et al., 2013) While this methodology may overestimate true carrier frequency and does not exclude patients with a history of melanoma, it should bias our results toward the null hypothesis. Further, while these patients are not always genotyped in the same way as our analysis, we do believe that it should be a reasonable surrogate to determine carrier estimate in the general population. Over 15 million individuals were screened between 1996 and 2000, with 1211 individuals noted to have some form of hyperphenylalanemia (Table 1). The calculated incidence of PKU/hyperphenylalanemia is 1/12,903 in Caucasian individuals in the United States, with a carrier frequency of 1/56.8 or 1.76%.

We obtained access to three separate melanoma repositories of germline whole exome sequencing data through dbGaP: phs000178.v9.p8, phs000452.v2.p1 and phs000933.v1.p1. The samples were restricted to those individuals listed as white/Caucasian, if this information was available. In total, 814 melanoma cases were identified. The BAM/SAM files were accessed through dbGaP, visualized using Integrated Genome Viewer (<http://software.broadinstitute.org/software/igv/>) and examined for germline *PAH* variants. Determination of potential pathogenicity in phenylketonuria and/or hyperphenylalanemia was achieved by cross-referencing mutation databases, including BIOPKU (<http://www.biopku.org/>), The Human Gene Mutation Database and/or literature searches for reports of particular mutations. Additionally, rare, uncharacterized variants were evaluated

for potential pathogenicity using various mutation and splice site analysis tools, including mutationtaster.org, mutationassessor.org and Human Splicing Finder (<http://www.umd.be/HSF3/>). Mutations were considered pathogenic if they were described in a published report of PKU and/or hyperphenylalanemia or listed in BIOPKU. Additionally, nonsense mutations not previously described were included. Using the BIOPKU database, we also included information on the percent activity of the mutant alleles and their most common patient phenotype.

We tested the hypothesis that melanoma patients have a greater carrier frequency compared to the general population using a one-sided t-test. This was done for each of the three populations separately, as well as all three combined. A p-value less than 0.05 was considered statistically significant.

Analysis of the three datasets revealed known and/or obligate (i.e. previously not reported nonsense mutations) disease-causing mutations associated with either PKU or hyperphenylalanemia in 29 individuals, with a carrier frequency of 3.56% in the three datasets combined. Individual dataset frequencies are shown in Table 2. Comparison to the established Caucasian frequency of hyperphenylalanemia/PKU carriers results in a relative enrichment of 2.02, or about twice as high as the general population (p-value= 3.4×10^{-5}). Statistical significance was achieved in 2 individual datasets (Table 2). Specific mutations identified are in Table 2, with one variant of unknown significance with high likelihood of pathogenicity (E330K).

Our analysis demonstrates an enrichment of phenylketonuria/hyperphenylalanemia carriers in melanoma cohorts. This enrichment suggests that possessing one mutated copy of the *PAH* gene results in an increased risk for melanoma, and known carriers may benefit from regular screening for melanoma to improve early detection. The particular *PAH* mutations identified are scattered throughout the protein, without preponderance within one region of the gene.

This is the first study to suggest an association between *PAH* and melanoma. Although this association was not described in previous genetic studies of melanoma, including large genome wide association studies, (Law et al., 2015) it is not surprising, given the rarity of individual mutations in this gene in the population as a whole. Genome wide association studies are not designed to detect many extremely rare, but deleterious, mutations in a single gene. Although genome wide association studies are well powered to detect many variants, studies like this emphasize the importance of biologically-based candidate gene association studies, as it would not be expected that one individual single nucleotide polymorphism would have enough association with enough of these mutations to be identified in a genome wide association study.

Limitations in this study include that we were not able to obtain race information for all datasets and limit cases only to Caucasian individuals. However, given that mutations in the *PAH* gene are less frequently found in non-Caucasian individuals, this would likely have resulted in additional individuals being included in the frequency calculation, biasing towards the null hypothesis. This, along with other potential population genetic differences,

may also explain the differences observed between datasets. Further, while not all of the samples were obtained from United States based patients, the majority of samples were from United States based patients. The *PAH* carrier frequency does vary between countries as compared to the United States, but the newborn screening statistics are less readily available for other whole countries, and so comparison with those calculations is less feasible.

It should be noted that PKU carriers typically do not have different fasting levels of tyrosine in comparison to controls. However, after a phenylalanine load, they do have elevated phenylalanine to tyrosine ratios in the blood. (Verduci et al., 2002) This may have an inhibitory effect on pigment production (Farishian and Whittaker, 1980), with resultant increased melanoma risk, explaining the phenomenon that we are describing.

In conclusion, we have identified a novel gene (*PAH*) associated with increased risk for melanoma development. Future studies, including case-control studies using current sequencing methods, will be needed to have a better understanding of this finding and the mechanisms behind the association.

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ABBREVIATIONS

PKU	Phenylketonuria
PAH	Phenylalanine hydroxylase

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Significance

Genetic risk underpins a significant portion of the basis by which individuals develop melanoma, with more high-risk genes being recently discovered. However, many of the genetic risk factors predisposing to melanoma have not been characterized. Based upon a hypothesis relating phenylalanine hydroxylase gene mutations (the causative gene in phenylketonuria) and reduced pigmentation with melanoma risk, we found a two-fold enrichment of phenylketonuria/hyperphenylalanemia carriers in melanoma patients. This represents a novel gene associated with melanoma risk.

Table 1

Estimation of carrier frequency based upon Newborn Screening Data (1996–2000)

Year	White births	Classical PKU	Variant (clinically significant)	Variant (not clinically significant)	Total affected individuals	Frequency of disease	Carrier frequency I
1996	3,096,244	134	38	60	232	0.0000749	0.0173
1997	3,072,640	143	38	66	247	0.0000804	0.0179
1998	3,122,121	120	56	45	221	0.0000708	0.0168
1999	3,136,338	144	75	39	258	0.0000823	0.0181
2000	3,198,747	147	58	48	253	0.0000791	0.0178
Total	15,626,090	688	265	258	1,211	0.0000775	0.0176

I Carrier frequency calculated using formula (Disease frequency⁴)(1/2). Data abstracted from: http://genes-r-us.uthscsa.edu/newborn_reports.

Table 2

Mutations reported in individual datasets and frequency rates

Dataset	Mutations reported	Average % PAH activity of mutant allele ¹	Most frequent patient phenotype with this allele ¹	Frequency	p-value
phs000178.v9.p8 (TCGA)	c.30C>G (G10=) (Dobrowolski et al., 2010)	N/A	N/A	18/447 (4.03%)	1.0 × 10 ⁻⁴
	T22K (2001)	N/A	N/A		
	c.168+5G>A, (Keil et al., 2013)	N/A	cPKU		
	H100R (Mallolas et al., 1999)	N/A	N/A		
	A104D (Keil et al., 2013)	27%	mPKU		
	V230I (Mallolas et al., 1999)	63%	MHP		
	R243* (Keil et al., 2013)	N/A	cPKU		
	R252W (Keil et al., 2013)	0%	cPKU		
	L311P (Pey et al., 2007)	1%	cPKU		
	L348V (Keil et al., 2013)	35%	mPKU		
	Y356H ²	N/A	N/A		
	E381* (novel obligate variant) ³	N/A	N/A		
	R408W (4 instances) (Keil et al., 2013)	2%	cPKU		
	c.1315 + 1G>A (Keil et al., 2013)	N/A	cPKU		
phs000452.v2.p1 (Broad)	c. *19G>T (Santana da Silva et al., 2003)	N/A	N/A	6/234 (2.56%) (7/234 (2.99%))	0.17 (0.073)
	T22K (2001)	N/A	N/A		
	R68S (Keil et al., 2013)	68%	mPKU		
	V245A (Pey et al., 2007)	50%	MHP		
	A300S (Keil et al., 2013)	31%	MHP		
	R408W (Keil et al., 2013)	2%	cPKU		
	R408Q (Pey et al., 2007)	46%	mPKU		
	(E330K) ⁴	N/A	N/A		
phs000933.v1.p1 (Yale)	I65V (Muntau et al., 2002)	N/A	mPKU	5/133 (3.76%)	0.037
	R243* (Keil et al., 2013)	N/A	cPKU		
	L348V (Keil et al., 2013)	35%	mPKU		

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Dataset	Mutations reported	Average % PAH activity of mutant allele ¹	Most frequent patient phenotype with this allele ¹	Frequency	p-value
	T380M (Mallolas et al., 1999)	28%	MHP		
	c.1315+1G>A (Keil et al., 2013)	N/A	cPKU		

¹Information obtained from <http://www.biopku.org>

²This variant was listed in the BIOPKU database (www.biopku.org) as communicated to the PAHdb through personal communication and was found in individuals in the BIOPKU database.

³Please note that this mutation is interestingly seen in the germline sequence in this patient but not in the tumor (present in a lower than expected frequency but in both reading directions in the germline samples). This may be explained by tumor revertant mosaicism that has been reported previously in breast cancer with germline mutations in *BRCA1* gene. (Kotoula et al., 2017)

⁴E330K is a variant noted at the site of a previously noted pathogenic variant (E330D), and this is a site of iron binding within the protein (Andersen et al., 2002) We, therefore, think that it is likely pathogenic, and have included calculations in parentheses for both frequency and statistical significance within this dataset.

N/A Not Available in BIOPKU Database